Probing Local Mobility in Carbonic Anhydrase: EPR of Spin-labelled SH Groups Introduced by Site-directed Mutagenesis

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Cloned human carbonic anhydrase, HCAII, and mutants thereof have been investigated by spin-probing methods during its unfolding caused by guanidine•HCl. The spin-probe, *N*-(2,2,5,5-tetramethyl-1-yloxypyrrolidin-3-yl)iodoacetamide, has been regiospecifically introduced into cysteines by site-directed mutagenesis in various positions of the protein structure. Here we focus on EPR spectra of three different spin-labelled enzymes at various guanidine•HCl concentrations (at equilibrium). The following spin-labelled mutants are discussed: W16C/C206S, W97C/C206S and F176C/C206S. The EPR spectra of the three mutants differ markedly at low guanidine•HCl concentration (0–1 mol dm^{-3}) particularly within the series W97C/C206S and F176C/C206S, showing the characteristic anisotropic slow motional features. The W16C/C206S label position is much more mobile in the folded structures. The rotational correlation times reflect the local environment of the spin-probe in the folded enzyme: in W97C it is located in the core, near the active centre, in W16C and F176C at more peripheral positions. All samples gave EPR spectra characteristic of a 'free' unfolded protein chain at guanidine-HCl concentrations of *ca.* 3 mol dm^{-3} and above, and could be characterised by using one component in lineshape simulations. The spectra of the W97C/C206S and F176C/C206S samples in low concentrations of guanidine•HCl (between *ca.* 0.1 and 2.0 mol dm^{-3}) could only be reproduced in simulations by introducing several components associated with rather different rotational correlation times. This seems to imply the co-existence of at least two dynamic structures in equilibrium during the intermediate stages of the unfolding process. It is compatible with earlier suggestions of a folding intermediate based on optical characterisation.

Recently, EPR in combination with spin-probing methods have attracted increased attention in studies of biological macromolecular systems.[1] This arises primarily for two reasons. Firstly, in protein engineering it has become possible to introduce spin-probes at almost any position in a protein chain by site-directed mutagenesis followed by labelling of selected functional units such as SH groups. In this way it is possible to probe the mobility and structure by measuring magnetic interactions of the probe with the local environment in a range of timescales otherwise inaccessible by other single experimental methods (typically μs to ns). Secondly, modern commercial EPR, double resonance and time resolved spectrometers have high sensitivity which permits investigation down to nanomolar concentrations, and thus the systems in question can be studied under biologically active conditions.

Cloned human carbonic anhydrase, HCAII, and mutants thereof unfold through two distinct transitions when the guanidine•HCl (Gu-HCl) concentration is raised from 0 to 5 mol dm^{-3}, indicating the existence of a folding intermediate.[2,3] The transitions have been established by recording the change in absorbance at 292 nm which depends on exposure of buried tryptophans to solvent during the unfolding process. To gain further information of the local motions and structural changes at various positions in the protein during the unfolding, amino acids at selected positions in the protein chain were substituted to cysteine by site-directed mutagenesis. The amino acids were selected to represent three different local environments in the protein; (i) deeply buried in the hydrophobic core (pos. 97, near the active centre), (ii) near the surface but still unexposed (pos. 176), (iii) in the flexible N-terminal region (pos. 16). A spin-probe, *N*-(2,2,5,5-tetramethyl-1-yloxypyrrolidin-3-yl)iodoacetamide, can then be attached at the SH group as we have done previously with the native enzyme containing one single cysteine.[4] (In the mutants, the naturally occurring cysteine in position 206 is replaced first by serine.) By a special purification method only the proteins retaining their enzymatic activity after the labelling were studied here.

Here we present optical absorption data and EPR spectra of three spin-labelled double mutants, namely, W97C/C206S, W16C/C206S and F176C/C206S.* The changes in the ratio $A_{292}:A_{260}$ indicate the existence of a folding intermediate at Gu-HCl concentrations of *ca.* 0.5–2.0 mol dm^{-3} as has been suggested previously.[3] The local structure and mobility are discussed qualitatively from the appearance of the EPR spectra. In an attempt to understand more details of the spectral changes occurring during the unfolding process we have performed simulations of slow-motional spectra. It is shown how the lineshapes of the labelled mutants in the folded states can be reproduced fairly well using one component and a simple diffusion model, however, several components seem necessary in building up the simulated spectrum at low Gu-HCl concentrations, particularly for the two mutants in which the spin label is immobilized in the folded protein structure.

To illustrate the discussions in the following sections a schematic drawing of the polypeptide backbone of human carbonic anhydrase is shown in Fig. 1.

Results and Discusion

Optical absorption.—The change in absorbance at 292 nm is

* Abbreviations: W, tryptophan; C, cysteine; S, serine; F, phenylalanine. Thus, W97C means that the tryptophan in position 97 has been replaced by a cysteine, *etc.*

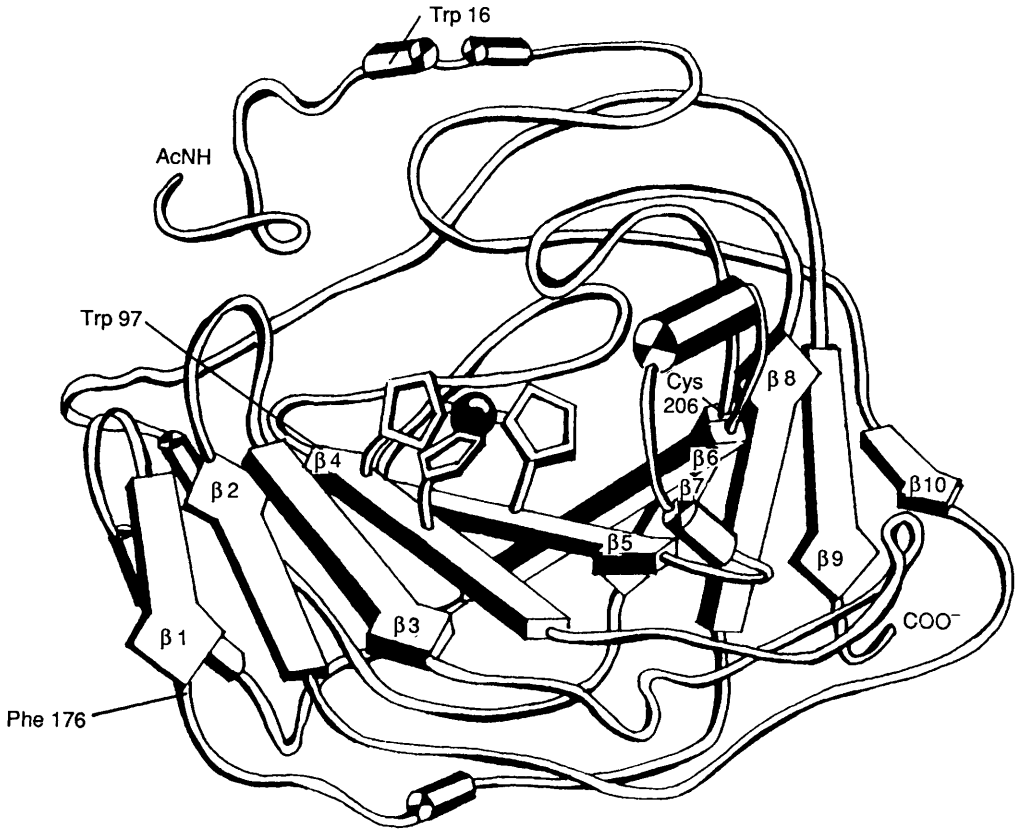

Fig. 1 Schematic drawing of the polypeptide backbone of human carbonic anhydrase II. Positions of amino acids and substitutions are indicated.

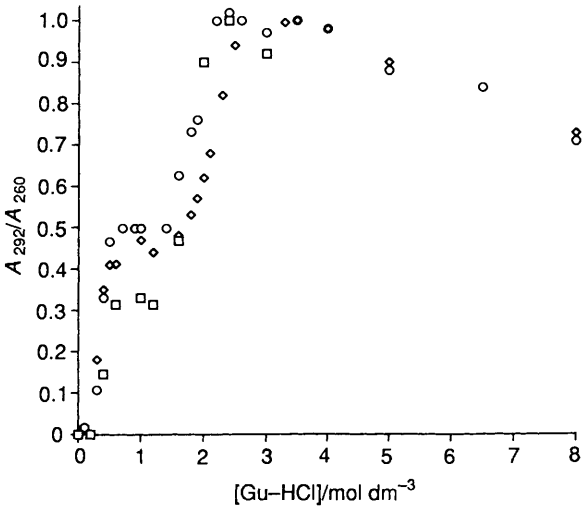

Fig. 2 The fractional change of the ratio of the absorption at 292 and 260 nm is plotted *vs.* Gu-HCl concentration, reflecting the degree of exposure of tryptophans to solvent for the three different spin-labelled mutants during the unfolding process: □, W97C/C206S; ◇, W16C/C206S; ○, F176C/C206S

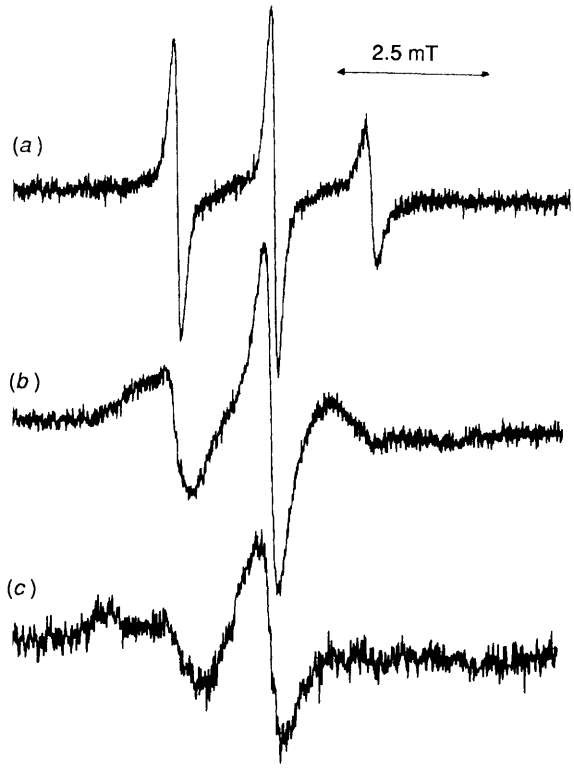

Fig. 3 EPR spectra of the three spin-labelled mutants in the native folded state (before denaturation): (*a*) W16C/C206S; (*b*) F176C/C206S; (*c*) W97C/C206S

presented in Fig. 2 as the ratio $A_{292}:A_{260}$ where A_{260} serves as an internal standard for protein concentration.[2] This parameter reflects the degree of exposure of buried tryptophans to solvent at various states of unfolding, and a series of other mutants has been discussed recently.[3] There are two distinct transitions and the enzymatic activity has been shown to be related to the first.[3]

The occurrence of two transitions has been suggested to imply a folding intermediate. Since there are seven tryptophan residues in the native protein the parameter given by the $A_{292}:A_{260}$ ratio reflects a global conformational change of the three-dimensional structure. In the following it will be shown how the introduction of spin probes gives further information in terms of local structure and mobility, particularly at low Gu-HCl concentrations.

EPR of the Folded Structure.—EPR spectra of the spin probe attached to the three mutants in the absence of any denaturat-

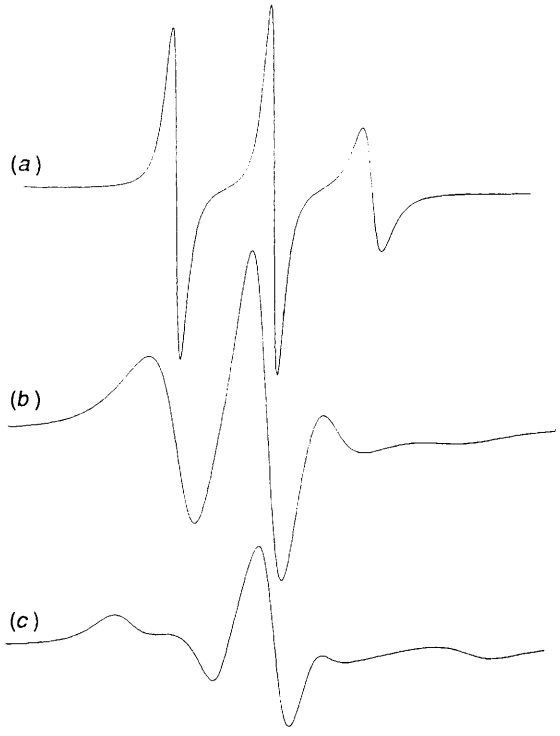

Fig. 4 Simulations of the spectra shown in Fig. 3(*a*)–(*c*). The hyperfine tensor and *g*-tensor of the rigid structure is given in the Experimental section. Diffusion parameters are given in the text.

ing agent, Gu-HCl, are shown in Fig. 3(*a*)–(*c*). There is a dramatic difference in the appearance of spectral anisotropic features characteristic of slow motion which reflects the local environment at the position of the spin probe in the protein molecule. Simulations of the spectra are shown in Fig. 4(*a*)–(*c*), and the relationship between the mobility and location of the label in the protein structure is discussed below.

The spectrum of W16C/C206S [Fig. 3(*a*)] can be characterised by a simulation [Fig. 4(*a*)] with the rotational diffusion tensor components; $d_{xy} = 2.4 \times 10^8$ s^{-1} and $d_{zz} = 4.2 \times 10^8$ s^{-1}. This is characteristic of a 'nearly free' polymer segment and consistent with the position of Trp 16; located as a part of an aromatic cluster at the *N*-terminal region,[5] that is, in the very peripheral part of the globular protein structure.

Trp 97 is situated at the end of β-strand No. 4 and is completely buried in the native state.[5] The EPR spectrum of W97C/C206S is also the one showing the most rigid character. The spectrum [Fig. 3(*c*)] can be simulated [Fig. 4(*c*)] using a rotational diffusion tensor $d_{xy} = 0.23 \times 10^8$ s^{-1} and $d_{zz} = 0.46 \times 10^8$ s^{-1}, that is, the motion of the spin probe is about one order of magnitude slower compared with the W16C/C206S case.

Phe 176 is situated close to β-strand No. 1 at the surface of the molecule, but the side chain points towards the interior of the protein.[5] Thus, it can be regarded as intermediate with respect to the interior and the outer surface of the protein. The folded state also shows a very complicated EPR spectrum [Fig. 3(*b*)] which seems to be composed of several components both of slow motional character and from more rapidly tumbling species. The attempted simulation shown in Fig. 4(*b*) uses only one species with the rotational diffusion tensor $d_{xy} = 0.45 \times 10^8$ s^{-1} and $d_{zz} = 0.90 \times 10^8$ s^{-1}.

Spectra at the Intermediate Unfolding Region.—With gradual increase of the concentration of Gu-HCl, there is a dramatic change in the EPR lineshape of the mutants W97C/C206S and F176C/C206S which showed the characteristic slow-motional spectra in the folded state.* Minor changes were found associated with W16C/C206S. This is consistent with the discussions above concerning the local structure and environment at the probe position (internal to external). W16C/C206S is located at the protein surface and is exposed to solvent during all stages of folding–unfolding, whereas in other variants the probe is more or less buried in the protein and should be more sensitive to unfolding.

The spectra of F176C/C206S in the range 0.1–0.5 mol dm^{-3} are shown in Fig. 5. The spectrum recorded at [Gu-HCl] = 0.3 mol dm^{-3} clearly shows a double peak on the high-field side characteristic of two superimposed components having rather different (independent) diffusion parameters. The series of spectra were assumed to be a superposition of a slow and a rapid species having diffusion tensor components $d_{xy} = 0.45 \times 10^8$ s^{-1} and $d_{zz} = 0.9 \times 10^8$ s^{-1}, and $d_{xy} = 1.8 \times 10^8$ s^{-1} and $d_{zz} = 3.6 \times 10^8$ s^{-1}, respectively. The former was chosen to represent a folded structure and the rapid component was chosen to fit spectra recorded at [Gu-HCl] levels of *ca.* 1–1.5 mol dm^{-3}, that is, in the region in which a folding intermediate would appear according to the optical data. The simulations (Fig. 6) reproduce the experimental spectra qualitatively (the fractions of the components are indicated in the figure) and may support the suggestion of a folding intermediate. However, based on EPR findings alone, we would regard this conclusion as very tentative owing to the simplicity of the model as well as the possibility of other phenomena which could lead to alternative explanations.† The modelling is discussed below.

It is emphasized here that the introduction (superposition) of a narrow component has a very dramatic effect on the appearance of the overall spectrum since its amplitude predominates over the components of the slow-motional contributors. This leads to extraordinary complications in the determination both of rotational diffusion times and relative contributions of the slow-motional components. It is also very difficult to distinguish between components having similar dynamic parameters and, thus, between components associated with a folding-intermediate structure and a completely open random coil. In other words, the probes in the intermediate state reflect local environments that are more similar to the denatured than the native state. Moreover, by introducing several species in equilibrium we should, in principle, also take into account the rate of change from one state to the other since this could affect the apparent lineshape as well. For comparison, the rotational diffusion coefficient (D_R) of native bovine CAII (a protein enzyme of very similar structure) has been given as $(1.14 \pm 0.15) \times 10^7$ s^{-1} from fluorescence correlation spectroscopy,[6] and can be related to the diffusion of the globular protein structure. The local changes of protein structure are probably faster and should be subjected to a more stringent analysis involving equilibria of several components.

Above *ca.* 1.0–1.5 mol dm^{-3} Gu-HCl the EPR spectra of the spin-labelled mutants became less sensitive to changes of mobility. Two characteristic spectra of spin-labelled F176C/C206S are shown in Fig. 7, which correspond to a folding

* Only F176C/C206S is discussed here, W97C/C206S showed even slower motional character but the signal-to-noise level of the spectra was too poor to allow a meaningful comparison with simulations. The spectra were shown and discussed in connection with a poster session related to the publication of this communication.

† The case could be that the segment of the protein chain on which the probe is located can take two (for simplicity) distinct positions: one directed into the interior of the globular structure as expected for the native (non-mutant) enzyme;[5] the other pointing out and being exposed more or less to the solvent phase; interconversion between such states cannot be excluded.

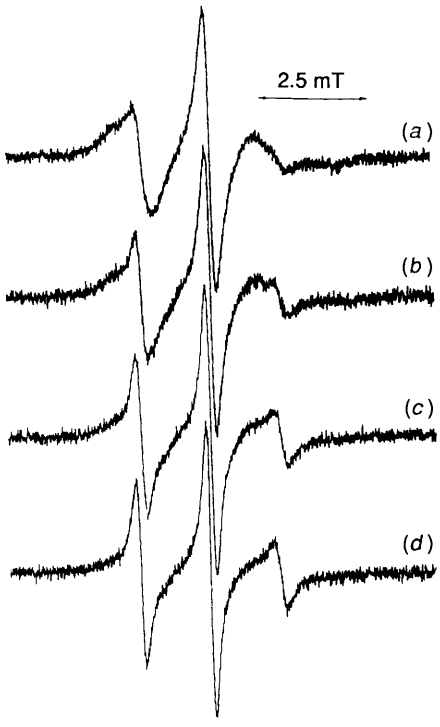

Fig. 5 EPR spectra of the spin-labelled mutant F176C/C206S during denaturation at various concentrations of denaturing agent (Gu-HCl): (*a*) 0.1; (*b*) 0.3; (*c*) 0.4; (*d*) 0.5 mol dm^{-3}

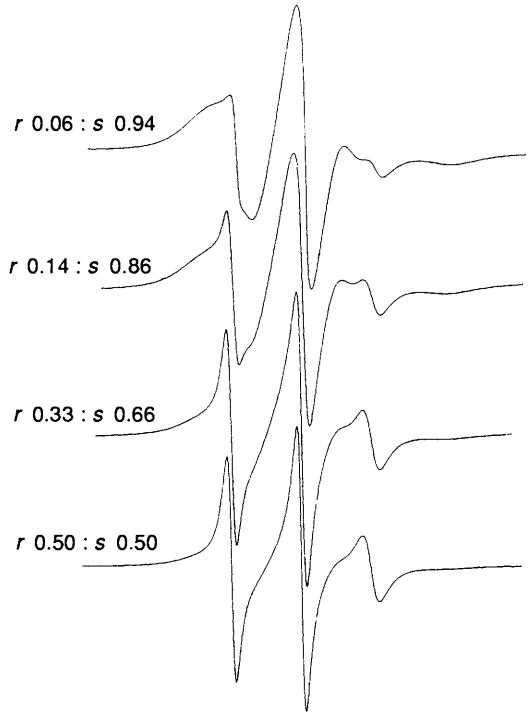

Fig. 6 Simulations of the spectra shown in Fig. 5 using superpositions of two components. The hyperfine tensor and *g*-tensor of the rigid structure are given in the Experimental section. Diffusion parameters are given in the text. *r* and *s* are the rapid and slow component respectively, as a fraction of the total integrated intensity: *r*, d_{xy} = 1.8×10^8 s^{-1}, d_{zz} = 3.6×10^8 s^{-1}; *s*, d_{xy} = 0.45×10^8 s^{-1}, d_{zz} = 0.90×10^8 s^{-1}.

intermediate state (1.6 mol dm^{-3}) and an unfolded state (3.0 mol dm^{-3}) on each side of the second transition obtained from the optical data (Fig. 2). The spin-labelled mutant W97C/C206S showed similar spectra at the same Gu-HCl concentrations, whereas the W16C/C206S variant showed a much less

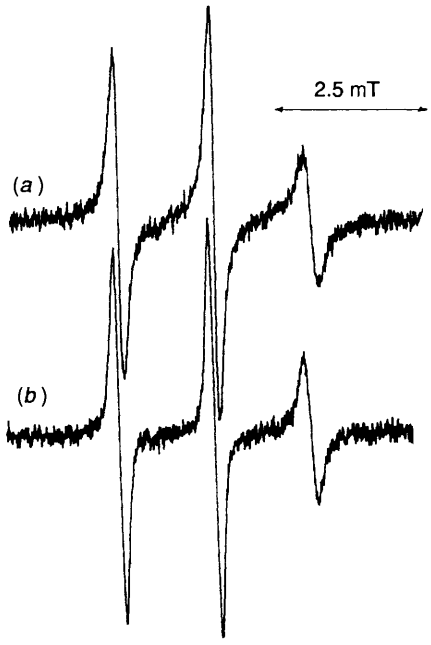

Fig. 7 EPR spectra of the spin-labelled mutant F176C/C206S denatured at (*a*) 1.6; (*b*) 3.0 mol dm^{-3}, Gu-HCl

pronounced difference between the intermediate and unfolded states. Since the changes in the EPR lineshapes are small it is not evident how these data should be modelled in simulations; the apparent small increase in mobility of the spin-probe could equally well be modelled by using a single species with different dynamic parameters, or by an equilibrium between several species of different dynamical parameters. We hope to be able to correlate the EPR data with results of other methods, such as CD (circular dichroism), in the near future and will return to these issues in forthcoming studies.

In conclusion, the EPR signature of the folded structure is strongly correlated to the location of the probe. An EPR characteristic of a rigid spin-probe is obtained when labelled in the rigid hydrophobic core. More solution-like EPR spectra are obtained for the mutant labelled at one terminus of the protein chain (outer region). The EPR spectra of the protein forms before the first transition (measured by optical absorption) can be modelled assuming an equilibrium between a rapidly moving and a slowly moving component. This may reflect an exchange between a folded structure and a folding intermediate. Above the first transition, *ca.* 1.5 mol dm^{-3} Gu-HCl, the associated EPR spectra are less sensitive to Gu-HCl concentration. Therefore, the characterisation of the transition: folding intermediate → random coil, is more difficult to establish solely from EPR data. Although there are apparent changes in the EPR lineshapes indicating the presence of a more mobile species above *ca.* 3.0 mol dm^{-3} the EPR lineshape data are not sufficiently sensitive for us to discern an equilibrium between several distinct species, or a sole species gradually changing.

Experimental

Sample Preparation.—Mutants of human carbonic anhydrase II were constructed by site-directed mutagenesis.[7] The expression plasmid pACA was used for the production of the enzyme variants. All enzyme variants were purified by affinity chromatography.[8] Three double mutants were prepared, W16C/C206S, W97C/C206S and F176C/C206S. A spin-probe, *N*-(2,2,5,5-tetramethyl-1-ylooxypyrrolidin-3-yl)iodoacetamide, was used to label the single SH group in each mutant. The W16C/C206S mutant (34 μmol dm^{-3}) was allowed to react with the spin probe (340 μmol dm^{-3}) in 0.1 mol dm^{-3} Tris–H_2SO_4,

pH 7.5, at 23 °C for 20 h. The reaction was stopped by adding 2-mercaptoethanol to a final concentration of 340 μmol dm^{-3}. The labelled W16C/C206S mutant was then dialysed towards 1 mmol dm^{-3} Tris H_2SO_4, pH 7.5. The W97C/C206S and F176C/C206S mutants (34 μmol dm^{-3}) were labelled (spin-probe concentration 680 μmol dm^{-3}) under denaturing conditions, 6 mol dm^{-3} GuHCl, and buffered with 0.1 mol dm^{-3} Tris–H_2SO_4, pH 7.5, 34 μmol dm^{-3} 2-mercaptoethanol at 23 °C for 12 h. The labelled enzymes were then reactivated by dilution to a final concentration of 0.85 μmol dm^{-3} and a GuHCl concentration of 0.2 mol dm^{-3}. The reactivation was allowed to proceed for 90 min. The active enzyme molecules were purified by affinity chromatography,[8] and in this way the excess of reagents was concomitantly removed. Dialysis was then performed as described for the W16C/C206S mutant. After dialysis all three enzyme variants were concentrated to a suitable concentration before EPR and UV measurements.

UV Measurements.—After incubation of enzyme (8.5 μmol dm^{-3}) in various concentrations (0–8 mol dm^{-3}) of Gu-HCl, buffered with 0.1 mol dm^{-3} Tris–H_2SO_4, pH 7.5, at 23 °C for 24 h, denaturation was monitored by measuring A_{292} as a function of Gu–HCl concentration. To compensate for variations in enzyme concentration the A_{292}:A_{260} ratio was determined, since A_{260} has been shown to be unaffected by complete denaturation.[9]

EPR Measurements.—The same enzyme samples as above were used for the EPR measurements. These were carried out employing a Bruker EPR spectrometer which consisted of a combination of the ER 200 and ESP 300 systems. The samples were introduced in standard flat cells for aqueous samples. A double ER4102ST cavity was used in most measurements. In the recording of EPR spectra, care was taken to avoid lineshape distortions which could arise from experimental conditions such as microwave saturation and overmodulation. Typically the modulation amplitude was set lower than half the linewidth of the $\Delta m_I = 0$ nitrogen hyperfine transition, typically 0.02 mT (fast motion)–0.09 mT (slow motion), at typically 1 mW microwave power. All experiments were carried out at room temperature, 19 ± 1 °C.

EPR lineshape simulations.—Programs developed by Freed and coworkers to account for slow-motional diffusion were employed.[10] The programs were modified to allow simple input/output, spectrum integration, addition/subtraction, etc. Most simulations were carried out using a 386 Acer laptop personal computer.

For the simulation of spin probes in motion it is desirable to have the EPR parameters of the rigid state. These were obtained by recording the spectrum of a frozen (water) solution of the spin probe. The following parameters were obtained and were used throughout the study: $g_x = 2.0084$, $g_y = 2.0056$, $g_z = 2.0020$; $A_x = 0.665$ mT, $A_y = 0.490$ mT, $A_z = 3.62$ mT. Intrinsic linewidths of 0.04–0.08 mT were used in the simulations of dynamic spectra.

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