Surface accessibility of aromatic residues in human lysozyme using photochemically induced dynamic nuclear polarization NMR spectroscopy

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Resonances in the photo-CIDNP spectrum of human lysozyme have been assigned to specific spin systems despite extensive spectral overlap using the two-dimensional photo-CIDNP COSY experiment Five of the 12 tyrosine, tryptophan and histidine residues of human lysozyme are found to be accessible to flavin dye in solution This result is in good agreement with surface accessibility calculations carried out on the human lysozyme crystal structure When amino acid differences are considered the photo-CIDNP results obtained for human lysozyme are in good agreement with results obtained previously for hen lysozyme

^{1}H-NMR CIDNP Lysozyme Surface accessibility

1. INTRODUCTION

The photochemically induced dynamic nuclear polarization (photo-CIDNP) technique has been used to study a large number of proteins in recent years [1,2] The photo-CIDNP effect in proteins arises from the reversible photochemical reaction of a flavin dye molecule with a tyrosine, tryptophan or histidine residue on the surface of the protein. The photo-CIDNP effect has been used to make resonance assignments in protein NMR spectra, probe the surface structure of proteins and obtain information about protein-lipid interactions [1–3]. The enhancement of resonances in the photo-CIDNP spectrum can be either positive (absorption) or negative (emission) Tyrosine, tryptophan and histidine each give a characteristic photo-CIDNP spectrum which makes possible the assignment of photo-CIDNP effects in protein spectra to specific types of residues [1].

Human lysozyme differs from hen lysozyme in about 40% of the amino acid sequence. In addition to the 51 amino acid differences human lysozyme contains an additional glycine residue inserted at position 48 in the sequence [4]. For this reason the numbering of homologous residues in hen and human lysozymes differs by one after residue 48. Several of the amino acid differences between the two proteins involve tyrosine, tryptophan and histidine residues. The X-ray structure of human lysozyme has been refined at a resolution of 1.5 Å [5,6] In the crystalline state hen and human lysozymes show extensive structural homology [5,6].

In this study the aromatic resonances which appear in the photo-CIDNP spectrum of human lysozyme are assigned with the aid of a two-dimensional photo-CIDNP COSY experiment [7] The results obtained here are compared with surface accessibilities calculated for the human lysozyme crystal structure and with results obtained previously for hen lysozyme.

2 MATERIALS AND METHODS

Human lysozyme (Sigma) was dialyzed to remove acetate, heated to 75°C for 10 min in D_2O to exchange labile protons and subsequently

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freeze-dried. Flavin I (7,8,10-trimethyl-3-carboxymethylisoalloxazine) was a generous gift from Dr F. Muller (Wageningen). NMR samples of 0.3 ml consisted of 1.4 mM protein and 0.4 mM flavin in 99 8% D_2O at pH 5.3. In some photo-CIDNP experiments 50 mM *N*-acetylglucosamine was added to the sample A fresh sample was used for each experiment

All experiments were performed at 360 MHz on a Bruker HX-360 NMR spectrometer controlled by an Aspect 2000 computer. The photo-CIDNP technique has been described [1,8] In the present experiments a saturation pulse method was used rather than the procedure of collecting light and dark spectra and subsequently subtracting these spectra [8] A 5 s train of saturation pulses is applied to the sample immediately before the 0.4 s laser pulse and the detection pulse. Thus, the only observable magnetization present during acquisition arises from photo-CIDNP enhancement. Good signal-to-noise ratios were obtained using between 1 and 32 scans

The two-dimensional photo-CIDNP COSY experiment has been described [7]. The pulse sequence used differs from the standard COSY RD–90°–t_1–90°–t_2 sequence [9] in the preparation period only. The relaxation delay (RD) of the COSY sequence is replaced by a 6 s delay for sample mixing followed by a 5 s train of saturation pulses and a 0 2 s laser pulse. Thus, the only magnetization which evolves during the t_1 period arises from photo-CIDNP enhanced resonances. This coherence is transferred to other coupled spins by the second 90° mixing pulse and magnetization is then detected during t_2 After two-dimensional Fourier transformation the F_1 domain represents resonances that exhibit a photo-CIDNP effect and the F_2 domain contains, in addition, resonances from nuclei that are coupled to the directly polarized ones. Thus, unlike a COSY spectrum the photo-CIDNP COSY spectrum is not symmetric about the diagonal.

In the experiment reported here 128 t_1 increments of 4K were collected. One scan was collected per t_1 increment. The transmitter offset was placed to the downfield edge of the aromatic region of the spectrum. The sweep widths in F_1 and F_2 were ± 714.3 and ± 3012 Hz, respectively.

3. RESULTS AND DISCUSSION

The photo-CIDNP spectrum of human lysozyme is shown in fig.1a. In the aromatic region of the spectrum several absorption lines arising from histidine and tryptophan residues and emission lines arising from tyrosine residues can be seen. In the aliphatic region of the spectrum absorption and emission lines arising from tyrosine, tryptophan and histidine H^{δ} resonances are also apparent. The addition of 50 mM *N*-acetylglucosamine (GlcNAc) to hen lysozyme samples has been found to lead to a 2-fold enhancement of the observed photo-CIDNP effects [1,2]. It has been suggested that this enhancement is related to the binding of flavin dye in the active site of lysozyme [1]. The displacement of flavin dye by the inhibitor GlcNAc may result in an increase in the number of flavin molecules available for CIDNP photoreaction [1]. A similar enhancement of photo-CIDNP effects is observed for human lysozyme (fig.1b). However, the presence of 50 mM GlcNAc does not give rise to significant shifts of any of the aromatic resonances in the photo-CIDNP spectrum of human lysozyme [10].

The aromatic region of the human lysozyme spectrum is composed of 45 overlapping

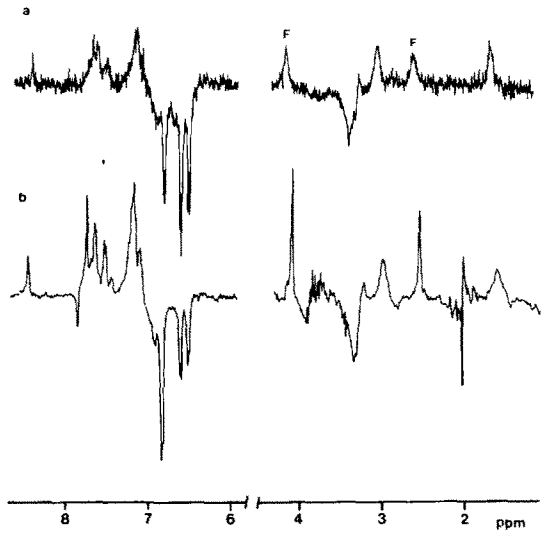

Fig 1 360 MHz photo-CIDNP spectrum of (a) 1 4 mM human lysozyme and (b) 1 4 mM human lysozyme plus 50 mM *N*-acetylglucosamine. Resonances arising from the enhancement of flavin dye are indicated (F)

resonances arising from the 6 tyrosine, 5 tryptophan, 2 phenylalanine and a single histidine residue. The absorption lines observed at 8.52 and 7.27 ppm in the photo-CIDNP spectrum arise from the $H^{\epsilon 1}$ and $H^{\delta 2}$ resonances of His-78. The other observed photo-CIDNP effects could arise from a number of tryptophan and tyrosine spin systems. The results of a two-dimensional photo-CIDNP COSY NMR experiment shown in fig.2 have been used to assign the remaining photo-CIDNP effects observed in the aromatic region to specific amino acid spin systems. The aromatic region of the NMR spectrum of human lysozyme has been assigned independently on the basis of NOE data [10,11,14]. The emission lines at 6.59, 6 69 and 6.89 ppm arise from tyrosine H^{ϵ}

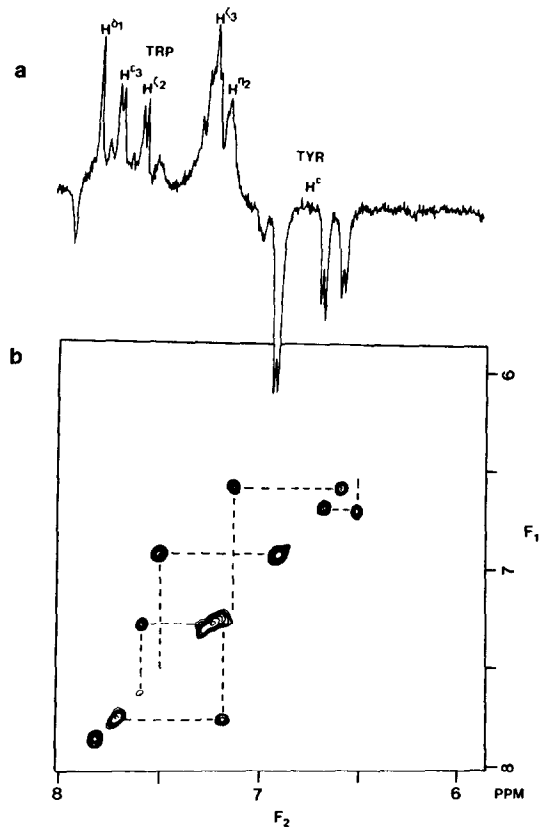

Fig 2 (a) Aromatic region of the 360 MHz photo-CIDNP spectrum of human lysozyme. Resonances arising from tryptophan and tyrosine residues are indicated. (b) Aromatic region of the 360 MHz two-dimensional photo-CIDNP correlated spectrum of human lysozyme.

resonances which are coupled to H^{δ} resonances at 7.16, 6.52 and 7.50 ppm, respectively. These spin systems have previously been assigned to Tyr-45, Tyr-63 and Tyr-124 [10,14]. The results of the photo-CIDNP experiments make it possible to distinguish unambiguously between the H^{δ} and H^{ϵ} resonances for these 3 tyrosine residues. In the absence of photo-CIDNP data this distinction could be made using pH titrations at high pH (pH > 10) or specific chemical modification studies [12]. Such experiments had not previously been carried out. It can be seen clearly in the 2-D map that the remaining absorption lines in the 7.15–7.80 ppm region of the spectrum arise from a single tryptophan spin system with $H^{\delta 1}$, $H^{\epsilon 3}$, $H^{\zeta 3}$, $H^{\eta 2}$ and $H^{\zeta 2}$ resonances at 7.78, 7 72, 7 16, 7.27 and 7 60 ppm, respectively This spin system has been assigned previously to Trp-34 [10,14] The complete assignment of the photo-CIDNP effects in the aromatic region of the spectrum is summarized in table 1.

The photo-CIDNP results presented above indicate that the Trp-34, Tyr-45, Tyr-63, His-78 and Tyr-124 residues of human lysozyme are all accessible to flavin dye in solution. These results can be compared with the accessibilities calculated for the aromatic residues of crystalline human lysozyme shown in table 2 (P.J. Artymiuk and C.C F. Blake, unpublished). The accessibility of the tyrosine hydroxyl groups in the crystal structure decreases in the order Tyr-63 > Tyr-45 > Tyr-124 > Tyr-20, Tyr-54 > Tyr-38. Thus, the 3

Table 1

Assignment of resonances in the photo-CIDNP spectrum of human lysozyme

Chemical shift (ppm)	Assignment	Origin of effect
8 52	His-78 $H^{\epsilon 1}$	D
7 78	Trp-34 $H^{\delta 1}$	D
7 72	Trp-34 $H^{\epsilon 3}$	D
7 60	Trp-34 $H^{\zeta 2}$	I
7.27	His-78 $H^{\delta 2}$, Trp-34 $H^{\eta 2}$	D
7 16	Trp-34 $H^{\zeta 3}$	I
6.89	Tyr-124 H^{ϵ}	D
6 69	Tyr-63 H^{ϵ}	D
6 59	Tyr-45 H^{ϵ}	D

D, direct polarization; I, indirect polarization

Table 2

Surface accessibilities calculated from the crystal structure

Residue	% Accessibility
Tyr-20	10
Tyr-38	2
Tyr-45	23
Tyr-54	10
Tyr-63	70
Tyr-124	18
His-78	67
Trp-28	0
Trp-34	57
Trp-64	24
Trp-109	14
Trp-112	16

Accessibilities are presented as the percentage of the area exposed in the protein compared to the area exposed in model tripeptides. Calculation for tyrosine are based on the exposure of O^{ζ}. Calculations for histidine are based on the exposure of $N^{\epsilon 2}$ and $N^{\delta 1}$ Calculations for tryptophan are based on the exposure of all atoms of the indole ring

tyrosine hydroxyl groups which are most accessible in the crystal are also the most accessible to flavin dye in solution. In the crystal structure the accessibility of the tryptophan indole rings decreases in the order Trp-34 > Trp-64 > Trp-112, Trp-109 > Trp-28. Thus, the tryptophan ring which is most accessible in the crystal is also found to be the most accessible to flavin dye in solution. The single histidine residue is found to be accessible in both the crystal structure and in solution.

The photo-CIDNP spectrum of hen lysozyme has been described in previous studies [1,2,8]. In hen lysozyme two tryptophan residues, Trp-62 and Trp-123, are found to be accessible to flavin dye in solution [8]. No photo-CIDNP effects involving tyrosine or histidine residues are found in hen lysozyme in contrast with the results reported above for human lysozyme. The differences observed between the photo-CIDNP spectra of hen and human lysozymes can be explained fully in terms of the amino acid differences found in the two proteins [4]. The two accessible tryptophan residues in hen lysozyme, Trp-62 and Trp-123, are replaced by tyrosine residues in human lysozyme. Photo-CIDNP effects are observed for the resonances of Tyr-63 and Tyr-124 in human lysozyme. The accessible tryptophan, tyrosine and histidine residues at positions 34, 45 and 78 in human lysozyme are replaced by phenylalanine, arginine and asparagine residues in hen lysozyme. The side chains of Phe-34, Arg-45 and Asn-77 are all accessible in the crystal structure of hen lysozyme [13]. Photo-CIDNP effects corresponding to Phe-34, Arg-45 and Asn-77 are not observed in the photo-CIDNP spectrum of hen lysozyme because these amino acid residues are not polarizable with the flavin dye [1]. Thus, the photo-CIDNP results obtained for hen and human lysozymes are consistent with a close structural homology for these two proteins in solution as had previously been observed for the crystalline states [5,6]

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