View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

The effect of pH and temperature on the structure of the active site of azurin from *Pseudomonas aeruginosa*

E.T. Adman+, G.W. Canters*, H.A.O. Hill and N.A. Kitchen

Inorganic Chemistry Laboratory, University of Oxford, Oxford OX1 3QR, England and *+Department of Biological Structure, University of Washington, Seattle, WA 98195, USA*

Received 26 May 1982

Azurin *Blue copper protein* *Pseudomonas Aeruginosa* *'H NMR spectroscopy* *Electron transfer* *Histidine*

1. INTRODUCTION

The unusual spectroscopic properties of the blue copper proteins, such as azurin and plastocyanin, have attracted much attention [1,2]. The success of spectroscopic methods in predicting the coordination environment of the copper could be assessed on publication of the crystal structures of a plastocyanin [3] and an azurin [4,5]. It appears that in poplar plastocyanin, the Cu-atom is coordinated by two histidines (residues 37 and 87), a cysteine (Cys 84) and a methionine (Met 92) in a distorted tetrahedral configuration [3]. Although the resolution of the crystal structure of azurin is somewhat less, the data suggest that the Cu is coordinated by two histidines (His 46 and 117), a cysteine (Cys 112) and a methionine (Met 121), again in a distorted tetrahedral configuration. Possible contributions from other ligands, e.g., from the peptide backbone, could not be completely excluded [5].

The information provided by the crystal structure determinations has been invaluable in allowing a more detailed interpretation of the spectroscopic data, not least that obtained from high resolution nuclear magnetic resonance spectroscopy. An important goal for all these studies is to relate the structures of the proteins to their functions. The question of the mechanism and control of the electron transfer in these proteins is still unanswered. *Pseudomonas aeruginosa* azurin can exist in a low and a high pH form the latter being at least 2 orders of magnitude slower in exchanging electrons cytochrome *c*-555 than the former. The redox potential of the 'inactive' form is 60 mV lower than that of the 'active' form, indicating a stabilization of the Cu(II) state towards high pH [6–8]. The transition from active to inactive state involves deprotonation of the histidine [6] which was shown in NMR studies [9–12] to participate in a slow proton-exchange process. In a recent NMR study [13] this residue has been assigned to His 35, which is adjacent to the ligand His 46 [4,5].

We wish to report the results of some NMR experiments on *Pseudomonas aeruginosa* azurin which may shed some light on the influence of temperature and pH on the structure of the active site of the protein.

2. MATERIALS AND METHODS

2.1. *Protein purification*

Pseudomas aeruginosa azurin was prepared according to standard procedures [14,15]. The purified protein was dialyzed against H_2O and stored at 4°C after lyophilization. Solutions of azurin (1–2 mM) were made up in 20 mM phosphate buffer in D_2O, through which nitrogen or argon had been passed.

2.2. *Sample preparation*

Samples of azurin were reduced by adding a solution containing 0.1 M NaOD and 0.1 M $Na_2S_2O_4$ in D_2O. Partial oxidation was achieved by titration with a dilute (1 mM), alkaline (1 mM NaOD) solution of $(NH_4)_2S_2O_8$ in D_2O. The dithionite and

* On leave from: the Department of Chemistry, University of Leiden, The Netherlands

Published by Elsevier Biomedical Press

00145793/82/0000–0000/$2.75 © 1982 Federation of European Biochemical Societies

persulphate solutions were kept at 0°C during the experiments to slow down decomposition. The pH of the solution was varied by adding small amounts of 1 M solutions of DCl or NaOD in D_2O. The pH of the sample was measured with a Pye-Unicam pH-meter, and quoted pH*-values are meter readings not corrected for the deuterium isotope effect. Azurin concentrations were measured spectrophotometrically by determining the absorbance of the oxidized species at 625 nm (ε_{625} = 5700 $mol^{-1} \cdot cm^{-1}$ [16]).

2.3. *NMR experiments*

NMR spectra were measured on the Bruker 300 MHz NMR Spectrometer of the Oxford Enzyme Group. Standard deconvolution procedures were used to process the FID signals [17]. A slight amount of dioxane was added to the sample to serve as an internal reference, but shifts are quoted with respect to TSS.

3. RESULTS AND DISCUSSION

3.1. *Assignment of resonances from the histidine and methionine ligands*

In order to analyse the effect of pH and temperature on the active site, the resonances of the Cu ligands have to be identified in the NMR spectrum. Fig.1 shows low-field and high-field portions of the ^1H NMR spectrum of Cu(I)azurin. The non-ligand histidine residues have been assigned in [9,11,12]. Those we associate with the C-2 and C-4 protons of the ligand histidines are labelled 1–4 in fig.1. They appear as singlets in Hahn spin-echo spectra and are most sensitive to slight oxidation of the copper (see fig.1). The chemical shift differences between the C-2 and C-4 proton resonances are unusually small (0.04 and 0.07 ppm) for both ligand histidines. In the ^1H NMR spectra of plastocyanin, the corresponding differences are 0.41 and 0.69 ppm, which, although larger than in the case of azurin, are also outside the usual range of 1.0–1.5 ppm [12]. We presume that binding of the histidines to the Cu(I) is responsible.

We had assigned resonances to 5 of the 6 methionines in azurin, with the sixth unaccounted for [12]. We now assign peak 5 (see fig.1) to the missing methionine on the basis of the following evidence: In the spectrum of the reduced azurin peak 5 overlaps with a resonance, R_4 [12,13], which is derived from the methyl group of an aliphatic residue which is far ($\geq$ 20 Å) from the Cu atom [13]. The data in fig.1 demonstrate therefore, that it is peak 5 and not R_4 that is affected by slight oxidation, indicating that peak 5 derives from a residue very close to the copper. Secondly, under conditions where peak 5 has its maximum intensity (low pH and temperature, vide infra) the area under R_4 and peak 5 together accounts for 5.7 ± 0.3 protons, compatible with the assignment of peak 5 to a methyl residue. Finally peak 5 has the appearance of a singlet as

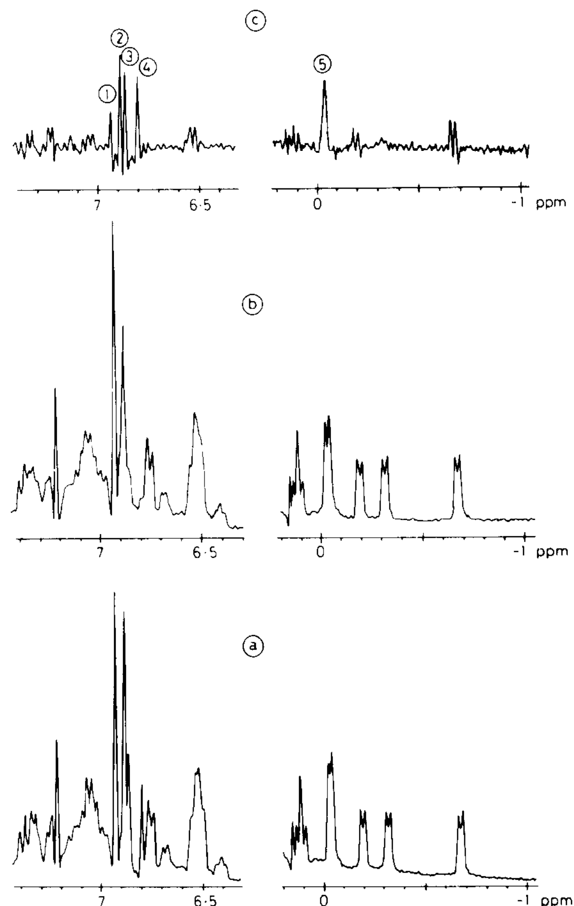

Fig.1. Low and high field portions of the 300 MHz ^1H NMR spectrum of a 1.5 mM solution of azurin in D_2O at pH* 6.5 and T = 50°C; phosphate buffer 20 mM: (a) conventional spectrum of 100% reduced solution; (b) as (a), ~ 1% oxidized; (c) difference spectrum (a)–(b); vertical display of the high field region twice that of the corresponding region in (a) and (b).

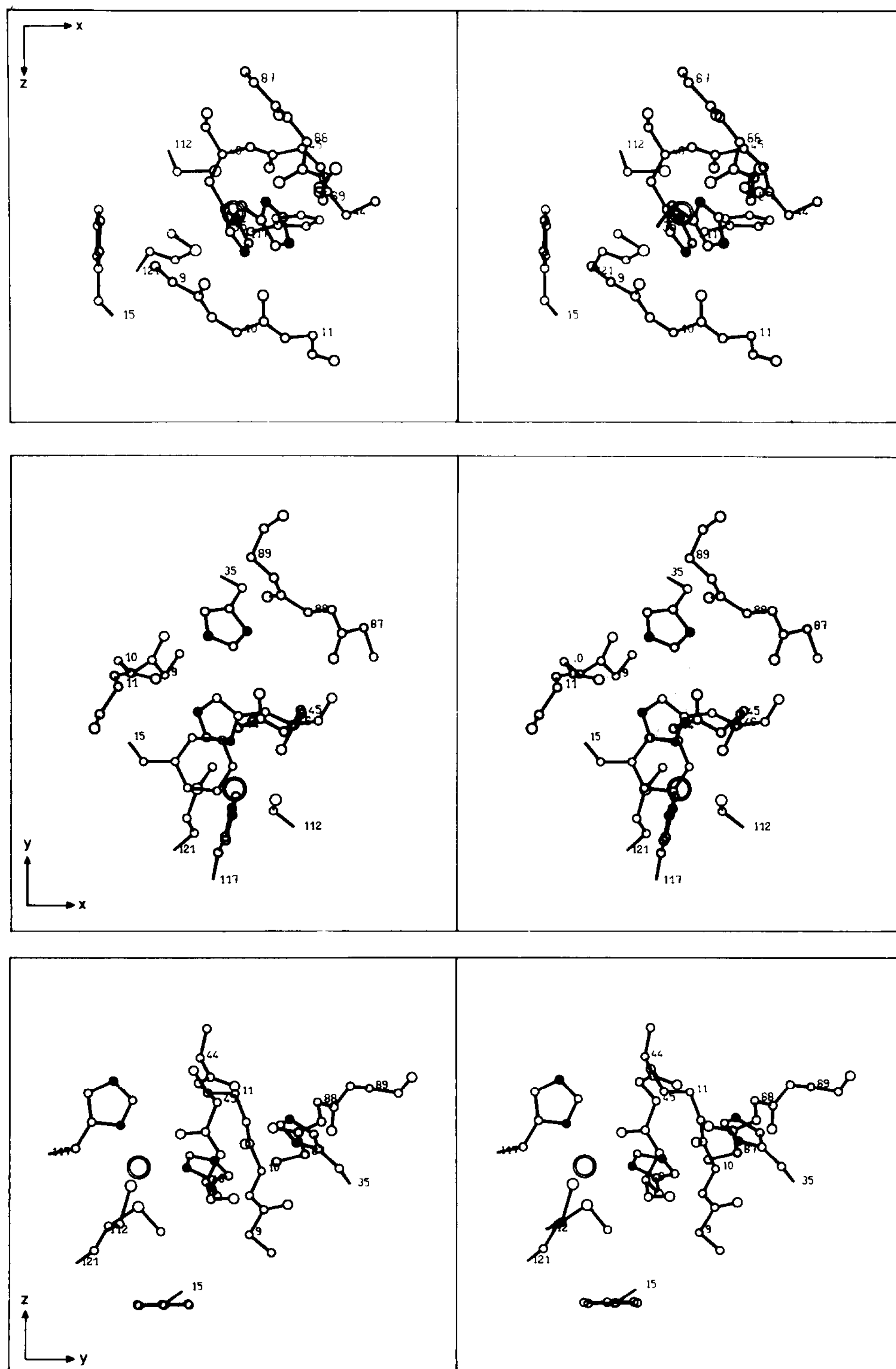

Fig.2. Stereoscopic projections of a portion of the molecular framework of azurin down 3 mutually orthogonal axes (x,y,z). The z-axis runs perpendicular to the plane of the aromatic ring of Phe 15. Crystallographic data from [4,5].

expected for a methyl methionine resonances. The CH_3-resonance from methionine amino acid residues usually occur in the region of 1.5–2.0 ppm and assignment of peak 5 to a methionine therefore implies a relatively large upfield shift for this resonance. A similar upfield shift has been reported for the ligand methionine in plastocyanin [18]. Inspection of the crystal structure of azurin [4,5] (fig.2) shows that the methyl group of Met 121 is sandwiched between the planes of Phe 15 and His 46, above the center of the Phe 15 ring. It is likely that the ring current of Phe 15 will shift the methyl resonance of Met 121 significantly upfield. A nuclear Overhauser experiment confirms the proximity of the residue associated with peak 5 to residue Phe 15 [13]. Thus we assign peak 5 to ligand Met 121 and peaks 1–4 to ligand His 117 and 46. We have no information on the resonances associated with the fourth ligand, Cys 112.

3.2. *pH and temperature effects*

There is a marked difference in the effect of pH and temperature on peaks 1–4 and peak 5. Whereas peaks 1–4 are unaffected by pH and temperature an increase in pH* from 4.6–8.2 causes (fig.3) a considerable reduction in the intensity of peak 5. In order to avoid denaturation at high temperature, and loss of resolution at low temperature, only a limited range of temperatures could be investigated. Nevertheless a decrease in intensity of peak 5, although not as large as when the pH is raised, accompanies an increase from 30–50°C. This can be seen in fig.4 by comparing the height of peak 5 with the height of neighbouring multiplets. In relation to those the intensity of peak 5 increases by ~ 30% when the temperature falls from 50–30°C. In contrast to this the peaks 3 and 4 show only small shifts (of the order of 0.01 ppm) when the temperature is changed from 20–50°C or the pH* is varied between 4.5–8.5.

The behaviour of peak 5 is typical of that of a residue undergoing increased motion at higher temperature and higher pH. Inspection of fig.2 shows, that rotation around, for instance the C_γ–S_δ or C_β–C_γ bond, will cause the terminal methyl group of Met 121 to move through the anisotropic field produced by the ring current of Phe 15. An order of magnitude estimate based on a simple model of the ring current dipolar field [19] shows that a librational motion around the C_γ–S_δ bond, with an amplitude of 10–20° may easily cause a variation in the chemical shift of the methyl protons of the order of 0.5 ppm. Broadening of the NMR signal ensues if the characteristic time of the libration is of the order of $\geq 10^{-2}$ s.

It is important to note that the conditions which induce broadening of the Met 121 CH_3-signal (high pH and temperature), are also the conditions which favour the formation of the redox-inactive form of the azurin [6]. As mentioned in section 1, the transition from active to inactive form has been shown to be connected with deprotonation of His 35. We suggest that the movement of Met 121, as reflected in the behaviour of peak 5, and the deprotonation of His 35, are interconnected. The fact that both processes occur on the same time-scale is consistent with this idea.

How are these two processes connected? It is clear that increased motion of Met 121 must go together with a change in the disposition, either dynamically or statically, of Met 121 with respect to the copper. Even rotation around the C_γ–S_δ bond, while not

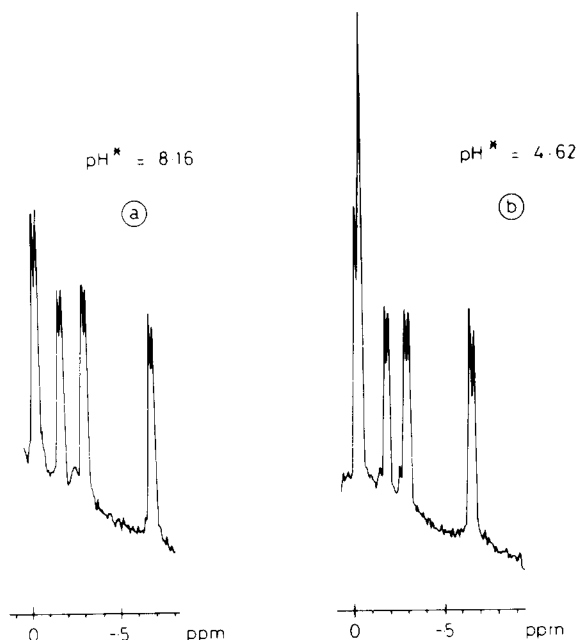

Fig.3. High field region of the 300 MHz ^{1}H-NMR spectrum of a 1.7 mM reduced solution of azurin in D_2O at T = 50°C containing 20 mM phospate buffer: (a) pH* 8.16; (b) pH* 4.62.

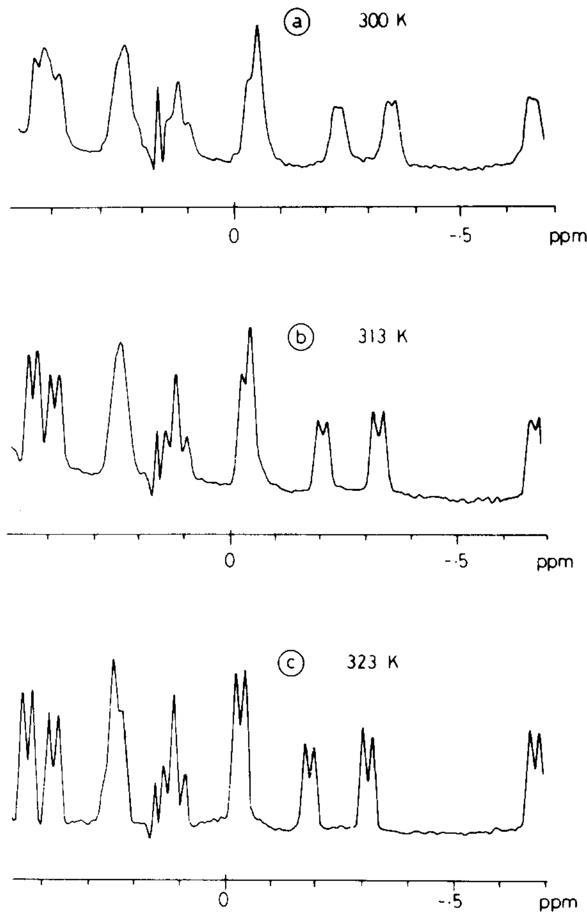

Fig.4. As fig.3; azurin 1.5 mM, pH* 6.5, T = (a) 27°C, (b) 40°C and (c) 50°C.

affecting the equilibrium Cu–S distance, would change the orientation of the sulfur σ and π orbitals with respect to the metal. The simplest explanation is that in the high-temperature, high pH-form the methionine is no longer coordinated to the copper. Fig.2 shows that an increase in the Cu–S (Met) distance might be compensated for, in part, by an increased interaction of the copper with the backbone of peptide 45 on the opposite side of the Cu. (Interaction may occur with either the carbonyl or the peptide nitrogen, since the crystallographic data allow for two orientations of the peptide, related to each other by a 180° flip-over.) This would presumably lead to a stabilisation of the copper(II) protein relative to the reduced form with a consequent reduction in the formal potential. Backbone involvement in the coordination of the Cu has been suggested [5,20]. It would provide an explanation for the occurrence of an unusual carbonyl resonance in the ^{13}C NMR spectrum of reduced azurin [10], which titrates with a pK ~ 7, the same pK with which His 35 titrates.

In considering how deprotonation of His 35 might be relayed to Met 121 it is important to realize that these data do not indicate whether the relay is effected via the Cu atom or through a change in peptide conformation. A conformational change in the tight loop containing 3 of the 4 ligand residues (roughly extending from residue 112–122) might change some of the constraints that preserve the tetrahedrally distorted conformation around the Cu [4]. Another possibility is that the deprotonation of His 35 is relayed to a neighbouring Cu ligand, His 46 in this case, and that this causes a redistribution of charge around the Cu leading to a change in the structure of its coordination sphere.

The question how the relay between His 35 and His 46 might operate requires additional comment. The unusual proton exchange kinetics of what we now know to be His 35, could be related to a process involving the simultaneous breakage and formation of different hydrogen bonds by the same histidine [6]. Fig.2 further shows [4,5], that His 46 and the carbonyl of Asn 10 are in a good position to form a hydrogen bond. An inspection of the density map, of azurin indeed reveals a continuous density from $N_{\varepsilon 2}$ of His 46 to the carbonyl oxygen of Asn 10. Fig.2 further shows that, with a slight reorientation around its C_α–C_β and C_β–C_γ bonds, His 35 could form hydrogen bonds with the backbone carbonyls of residues 88 and 44. According to the mechanism envisaged in [6], at high pH His 35 would deprotonate presumably at the $N_{\varepsilon 2}$ position, which would then be available for hydrogen bonding with His 46. Published ^{13}C NMR evidence [10], however, indicates that His 35 deprotonates at the $N_{\delta 1}$ and not at the $N_{\varepsilon 2}$ position, as required by this mechanism. Alternatively, it is possible that His 35, although less ideally placed than His 46, could compete with His 46 for hydrogen bonding with Asn 10 (see fig.2), especially when rotated slightly around its C–C and C_β–C_γ bonds. In this manner it could influence the charge distribution in His 46 and thus the charge distribution around the Cu. Since protonation of His 35 will affect its ability to form hydrogen bonds, it will also influence its

ability to compete effectively with His 46 for hydrogen bond formation.

According to this model the switch from redox-active to redox-inactive state involves some kind of shuttling motion of the imidazole ring of His 35. In one position it would be accessible to water molecules and protonation could take place; in other position it would be inaccessible for outside water molecules but would be available for hydrogen bonding. The slow proton exchange of His 35 then might be connected with this motion of the imidazole ring.

To summarize: the effect of temperature and pH on the NMR spectrum of *Pseudomonas aeruginosa* azurin suggests that the switch from the redox-active form to the redox-inactive species is initiated by deprotonation of His 35 and is accompanied by a lengthening, perhaps breaking of the Cu–S (Met 121) bond. We expect that a further study of the effects of temperature, pH and degree of reduction on the NMR spectra will provide more insight into the mechanism by which the change in redox activity is brought about.

ACKNOWLEDGEMENTS

We thank the SERC for a studentship to N.A.K. and EMBO for a short-term Fellowship to G.W.C. E.T.A. wishes to acknowledge support from NIH grant GM 13366 to L.H. Jensen. We thank the Department of Molecular Biophysics, Oxford University, for the use of its computing facilities in elaborating on the crystallographic data. This is a contribution from the Oxford Enzyme Group of which H.A.O.H. is a member.

REFERENCES

[1] Gray, H.B., and Solomon, E.I. (1981) in: Metal Ions in Biology (Spiro, T.G. ed) vol. 3, pp.1–39, Wiley, New York.

[2] Ulrich, E.L. and Markley, J.L. (1978) Coordin. Chem. Rev. 27, 109–140.

[3] Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324.

[4] Adman, E.T., Stenkamp, R.E., Sieker, L.C. and Jensen, L.H. (1978) J. Mol. Biol. 123, 35–47.

[5] Adman, E.T. and Jensen, L.H. (1981) Isr. J. Chem. 21, 8–12.

[6] Silvestrini, M.C., Brunori, M., Wilson, M.T. and Darley-Usmar, V.M. (1981) J. Inorg. Biochem. 14, 327–338.

[7] Wherland, S. and Pecht, I. (1978) Biochemistry 17, 2582–2591.

[8] Rosen, P. and Pecht, I. (1976) Biochemistry 15, 775–786.

[9] Hill, H.A.O., Leer, J.C., Smith, B.E. and Storm, C.B. (1976) Biochem. Biophys, Res. Commun. 70, 331–338.

[10] Ugurbil, K., Norton, R.S., Allerhand, A. and Bersohn, R. (1977) Biochemistry 16, 886–894.

[11] Ugurbil, K. and Bersohn, R. (1977) Biochemistry 16, 3016–3023.

[12] Hill, H.A.O. and Smith, B.E. (1979) J. Inorgan. Biochem. 11, 79–93.

[13] Adman, E.T., Canters, G.W., Hill, H.A.O. and Kitchen, N.A. (1982) in preparation.

[14] Ambler, R.P. (1963) Biochem. J. 89, 341–349.

[15] Parr, S.R., Barber, D., Greenwood, C., Phillips, B.W. and Melling, J. (1976) Biochem. J. 157, 423–430.

[16] Goldberg, M. and Pecht, I. (1976) Biochemistry 15, 4197–4208.

[17a] Ernst, R.R. (1966) Adv. Magn. Res. 2, 1–135.

[17b] Ferrige, A.G. and Lindon, J.C. (1978) J. Magn. Res. 31, 337–340.

[18] Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980a) Biochim. Biophys. Acta 591, 162–176; (1980b) Nature 283, 682–683.

[19] Becker, E.D. (1980) in: High Resolution NMR, pp. 73–74, Academic Press, London.

[20] Miskowski, V., Tang, S.,-P.W., Spiro, T.G., Shapiro, E. and Moss, T.H. (1975) Biochemistry 14, 1244–1250.