Hen Egg White Lysozyme: Carbon-13 Nuclear Magnetic Resonance Assignments and Dependence of Conformational Flexibility on Inhibitor Binding and Temperature†

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ABSTRACT: A total of 23 [^{13}C]methyl resonances of lysozyme is firmly or partially assigned. Much of the molecule is shown to be relatively inflexible. Thus, the inter-methyl distance ratios observed in the crystal between Val-93 and Leu-17 are conserved in solution, and also, much of the thermal dependence of the ^{13}C shift is shown to arise from the active site cleft region only and to be substantially removed upon binding of 2-acetamido-2-deoxy-β-D-glucopyranoside (Glc-N-Ac). $(Glc\text{-}N\text{-}Ac)_3$ does not significantly affect regions of the molecule other than those affected by Glc-N-Ac. Lysozyme denatures in a single step to give a new, structured state.

Although considerable ^{1}H NMR[1] assignment work has been carried out on hen egg white lysozyme [for a recent summary, see Poulsen et al. (1980)] together with assignment of non-protonated ^{13}C resonances in the aromatic and carboxylate regions (Allerhand et al., 1977; Shindo & Cohen, 1976), there have been no ^{13}C NMR assignments to date in the main aliphatic region of the spectrum. However, many single methyl carbon resonances, together with some others, are resolvable at high field (Figure 1). Their assignment makes available many new markers for future investigations, some in hitherto "invisible" regions of the protein. Also, their perturbations readily yield information on the extent of conformational change induced by ligand binding, and any unusual temperature dependence of a chemical shift probably reveals the admixture of a significantly different but thermally accessible local conformation. Such considerations prompted us to undertake a high-field ^{13}C NMR study of lysozyme, concentrating particularly on methyl resonances. Our assignments were substantially assisted by access to details of an X-ray crystallographic study by Artymiuck et al. (1979).

A longer term aim of this study was attempts to understand the reported unfolding of proteins in much more detail than has hitherto been possible. Lysozyme and ribonuclease A [see preceding paper (Howarth & Lian, 1984)] were chosen for contrast because the former has been claimed to unfold in one stage and the latter in several. In the event, we have found that neither protein is affected by denaturants in more than a minor way until a single denaturation transition to a new but structured state.

Experimental Procedures

Materials. Lysozyme from hen egg white (grade 1) was obtained from Sigma Chemical Co., dialyzed according to the method of Dobson (1975), and then lyophilized, the solid protein being stored at −5 °C. *N*-Bromosuccinimide (NBS) (Sigma Chemical Co.) was recrystallized 3 times from water. Praseodymium chloride (Aldrich Chemical Co.), lanthanum chloride (BDH Chemicals, Ltd.), and Glc-N-Ac (Sigma Chemical Co.) were used as supplied. $(Glc\text{-}N\text{-}Ac)_3$ was a generous gift of Prof. K. Hamaguchi, Osaka University, Osaka, Japan.

The reaction of lysozyme with iodine at pH 5.5 was carried out essentially as described by Norton & Allerhand (1976a). Its treatment with an equimolar amount of NBS was carried out according to the procedure of Norton & Allerhand (1976b).

Methods. DEPT ^{13}C{^{1}H} NMR spectra were obtained by the method of Doddrell et al. (1982) with overnight accumulation and an interpulse delay of 0.5 s. Other methods were as in the preceding paper.

Results and Discussion

Most of the observations concern ^{13}C resonances of methyl groups in the range δ 10–23, because many of these are resolved into single-carbon peaks. A ^{13}C{^{1}H} DEPT experiment confirmed that no peak in this range arises from a methylene group, and also showed that the peaks from δ 60 to 62 account for all the Ser-C$^\beta$ carbons. The two pD values chosen for study, 3.2 and 5.0, are ones at which self-association of lysozyme is known to be minimal.

This was confirmed by the observation that the C$^\gamma$ resonances of Trp-62 and Trp-63 remained separate at all temperatures used, for Shindo & Cohen (1976) have shown that these peaks merge at δ 110.8 when the protein self-aggregates.

Preliminary ^{1}H NMR Assignments and Single-Frequency Decoupling. The ^{1}H NMR spectrum of lysozyme has been extensively assigned, as summarized by Poulsen et al. (1980). This work has been of great value in the present task, not only because one would expect many of the same shift perturbations to show in the ^{13}C spectrum but also because, where it is possible to irradiate a single ^{1}H resonance selectively, the resonance of the ^{13}C nucleus bound to that proton will often be identifiable as a uniquely sharp peak in the resulting ^{13}C spectrum. The complete resulting assignments are listed in Table I.

In the course of checking previous ^{1}H assignments by truncated nuclear Overhauser enhancement (NOE) difference spectroscopy, we observed enhancement of a peak at δ 0.74,

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[1] Abbreviations: lysozyme, lysozyme from hen egg white; Glc-N-Ac, 2-acetamido-2-deoxy-β-D-glucopyranoside; DEPT, distortionless editing by polarization transfer; NMR, nuclear magnetic resonance; NBS, *N*-bromosuccinimide; NOE, nuclear Overhauser enhancement; rms, root mean square; δ_C and δ_H, ^{13}C and ^{1}H chemical shifts in parts per million relative to Me_4Si.

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Table I: ^{13}C NMR Chemical Shifts and Assignments of Some Methyl Carbon Resonances in Hen Egg White Lysozyme

peak no.[a]	δ (^{13}C)	resonance assignment[b]	δ (^{1}H)	confirmatory evidence for assignment[c]	sample conditions[d]
1	12.11	Ile-98 $C^{\gamma 2}$	−0.26[g]	differential line broadening, I_2 treatment, Glc-N-Ac and Pr^{3+} binding	pD 5.0, 50 °C
2 and 3	13.0–13.05	(?) Ile-78 $C^{\gamma 2}$ and another (?) Ile methyl group	0.86[h]		pD 5.0, 50 °C
4	13.05	(?) Ile-55 $C^{\gamma 2}$ or C^{δ}	0.91	Pr^{3+} binding	pD 5.0, 50 °C
5	13.61	Ile-88 C^{δ}	0.17[g]		pD 3.2, +Glc-N-Ac
6	14.05	Ile-98 C^{δ}	−0.01[g]	differential line broadening, I_2 treatment, Glc-N-Ac and Pr^{3+} binding	pD 5.0, 50 °C
7	14.28	Ile C^{δ} or $C^{\gamma 2}$	0.74		pD 3,2, +Glc-N-Ac, 45 °C
8	14.37	Met-105 C^{ϵ}	−0.01[g]	I_2 treatment, Pr^{3+} and Glc-N-Ac binding	pD 5.0, 50 °C
9	15.05	Ile-58 $C^{\gamma 2}$ or C^{δ}	0.91[h]	Glc-N-Ac binding, I_2 treatment	pD 5.0, 50 °C
10	15.73	Ile-88 $C^{\gamma 2}$	0.74	pH titration (pK_a = 5.7)	pD 3.2, +Glc-N-Ac, 45 °C
11	15.73	Ile-58 C^{δ} or $C^{\gamma 2}$	1.04[h]	I_2 treatment, Pr^{3+} binding	pD 5.0, 50 °C
13	16.49[e]	Met-12 C^{ϵ}	1.55[g]	photooxidation[f]	pD 5.0, 50 °C
14	16.49[e]	Leu-75 C^{δ}		$(Glc\text{-}N\text{-}Ac)_3$ binding	
16	16.86	Ala-95 C^{β}	1.55[g]		pD 5.0, 50 °C
18	17.43	Ala-110 C^{β}	1.38[g]	Pr^{3+} binding	pD 5.0, 50 °C
19	17.72	Ala-107 C^{β}	0.64[g]		pD 5.0, 50 °C
21	17.97	(?) Val-99 $C^{\gamma 2}$	1.20[g]		pD 5.0, +$(Glc\text{-}N\text{-}Ac)_3$, 40 °C
23	18.78	Leu-56 $C^{\delta 1}$	0.22[g]	Glc-N-Ac and Pr^{3+} binding	pD 3.2, 50 °C
25a	19.62	Leu-17 $C^{\delta 2}$	−0.67[g]		pD 3.2, +Glc-N-Ac, 45 °C
25b	19.62	Val-92 $C^{\gamma 2}$	0.43[g]		pD 5.0, 50 °C
27	19.93	Val-109 $C^{\gamma 1}$	1.04[g]	(?) I_2 oxidation, Pr^{3+} binding	pD 5.0, 50 °C
28 or 29	20.53	Val-99 $C^{\gamma 1}$	1.27[h]		pD 5.0, +$(Glc\text{-}N\text{-}Ac)_3$, 40 °C
28 or 29	20.53	Val-92 $C^{\gamma 2}$	0.64[g]		pD 5.0, 50 °C
36	22.26	Leu-8 $C^{\delta 1}$	−0.01[g]		pD 5.0, 50 °C

[a] Peak numbers are those shown in Figure 1. [b] Less definite assignments are indicated by (?). [c] Almost all assignments include single-frequency ^{1}H decoupling of a previously assigned ^{1}H peak at the indicated shift. [d] These conditions were used in order to minimize overlap in the ^{1}H spectrum. [e] Overlapping pair. [f] Photooxidation experiments indicated a Met methyl group at this chemical shift although the analysis was hampered by another methyl group resonating at this same frequency. [g] From Poulsen et al. (1980) and references cited therein. [h] From Delepierre et al. (1982).

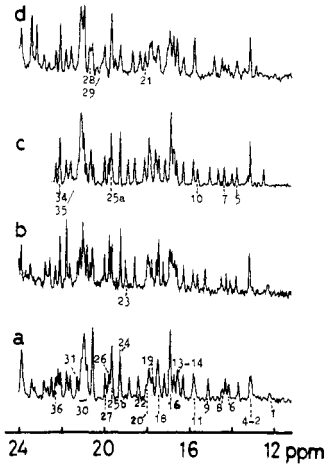

FIGURE 1: Peak numbering scheme. Region from δ 11.0 to 24.0 of the proton-decoupled ^{13}C NMR spectrum of lysozyme (6 mM in D_2O). (a) Free protein, pD 5.0, 50 °C; (b) free protein, pD 3.2, 50 °C; (c) +100 mM Glc-N-Ac, pD 3.2, 45 °C; (d) +12 mM $(Glc\text{-}N\text{-}Ac)_3$, pD 5.0, 40 °C. In this figure and in subsequent lysozyme spectra, the numbers indicate the position of individual methyl carbon resonances.

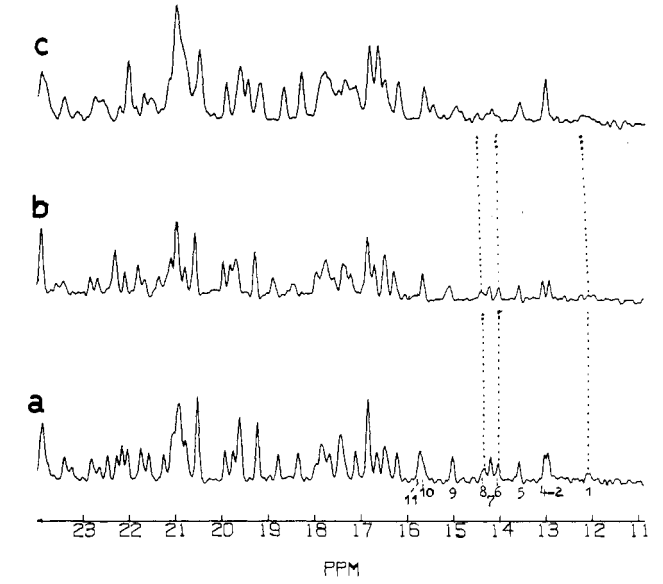

FIGURE 2: Spectral region from δ 11.0 to 24.0 of the proton-decoupled ^{13}C NMR spectrum of chemically modified lysozyme: (a) native, pD 5.2, 50 °C; (b) NBS treated, pD 5.4, 50 °C; (c) I_2 treated, pD 5.4, 50 °C.

in addition to other previously assigned peaks (Leu-8 $H^{\delta 1}$, Val-92 $H^{\gamma 2}$, and Met-12 H^{ϵ}), upon irradiation of Ile-88 H^{δ} at δ 0.17. We assign this peak to Ile-88 $H^{\gamma 2}$, as these are the only other nearby protons likely to give a resonance in this region of the spectrum. We also found that the relative NOE's between on the one hand Leu-17 $H^{\delta 1}$ and $H^{\delta 2}$ and on the other hand Val-92 $H^{\gamma 1}$ and $H^{\gamma 2}$, and Trp-20 and Leu-17 H^{α}, showed that their relative distances in the crystal are maintained in solution (Blake et al., 1967; Artymiuck et al., 1979).

pD Titration. Campbell et al. (1975) showed that above pD 5.0 the ^{1}H methyl resonances of Ile-98 become broadened, presumably because a local conformation or protonation change becomes relatively slow. We observed a similar broadening in the same pD range for carbon peaks 1 and 6 (numberings as in Table I and Figure 1), and we take this to support their assignment, via selective decoupling as Ile-98 C^{δ} and $C^{\gamma 2}$. Similarly, peak 10 titrates corresponding to a pK_a of 5.7, i.e., that of His-15. This confirms its assignment to Ile-88 $C^{\gamma 2}$, by analogy with previous ^{1}H work. A small shift is also noted in peak 8 (Met-105 C^{ϵ}, see below), peak 16 (Ala-95 C^{β}, lying at 920 pm from His-15), and also in a few other as yet unassigned methyl resonances, e.g., 15 and 30.

Chemical Modification. The conversion of Trp-62 to oxindolylalanine, described above, does not produce large changes in the ^{13}C spectrum (Figure 2b). This concurs with ^{1}H observations. Small shifts are, however, evident for peaks 9, 11, 12, 17, 20, 22, 23, and 35. Peaks 9 and 11 are assigned by other methods to the Ile-58 methyl. Also, peak 23 is assigned

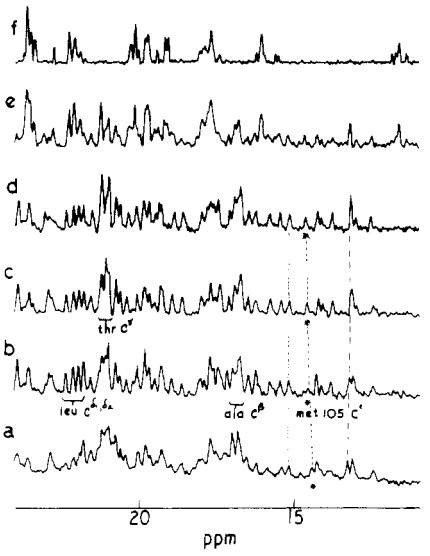

FIGURE 3: Effect of urea on the ^{13}C NMR spectrum of native lysozyme (pD 3.2, 7 mM in D_2O, 25 °C). Resonances marked with lines are assigned (left to right) as Ile-88 C^{γ_2}, Met-105 C^{ϵ}, and Ile-55 C^{δ}. (a) Native and (b) 1, (c) 2, (d) 4, (e) 6, and (f) 8 M urea.

below to Leu-56 C^{δ_1}. In the native protein, this atom lies at ca. 2000 pm from Trp-62. This may indicate that the Trp ring adopts a significantly different conformation upon oxidation so as to swing down into the vacant active site cleft, near to Ile-58 and Leu-56.

In contrast, the I_2 oxidation of Trp-108, which gives the Glu-35–Trp-108 ester, affects more methyl resonances (Figure 2c). Those that are assigned below are resonances 1 and 6 (Ile-98), 8 (Met-105), 9 and 11 (Ile-58), and 27 (Val-109 C^{γ_2}). These perturbations are all entirely consistent with the proposed oxidation, as these atoms lie from 420 to 1160 pm away from the affected Trp-108 ring

Jori et al. (1969) have shown that photooxidation with hematoporphyrin leads to the specific oxidation of Met-12. Upon performing this experiment, we observed a ca. 50% reduction in the intensity of peak 13/14. We therefore assign one component of this peak to Met-12 C^{ϵ}.

Binding of Pr^{3+}. The major binding sites common to La^{3+} and Pr^{3+} are known from previous studies (Poulsen et al., 1980). They are both close to Gln-57 and Leu-56. The ^{13}C spectra from δ 11 to 24 at pD 5.0 °C with each ion bound have been measured. The largest shift difference ($Pr^{3+} - La^{3+}$) is that of peak 23. This was assigned above by selective decoupling as Leu-56 C^{δ_1}; it shifts by δ −0.41 on addition of Pr^{3+}, relative to La^{3+}. The original ^1H assignment rested on a similar observation. Many other small methyl resonance shifts are also observed, notably in peaks 1 and 6 (assigned above to Ile-98), 4 (Ile-55), 8 (Met-105), 11 (Ile-58), 18 (Ala-110), and 27 (Val-109). These lie from 370 to 1070 pm from the Pr^{3+} binding sites. Hence, these shifts support the assignments proposed in other sections.

Urea Binding. Figure 3 shows the effects of adding urea to lysozyme. Many resonances are unaffected. However, as with ribonuclease A, a few show shifts at low urea concentration consistent with the binding of one or a few urea molecules with K_M = 0.5 M, at as yet undertermined sites. This binding also sharpens the resonances, presumably by removing some alternative minor conformation. No changes occur between 2 M and the onset of denaturation at 3.5 M. Above 3.5 M, as with ribonuclease A, the native state is in kinetically slow exchange with the denatured state, and the latter is clearly seen to be structured by the shift heterogeneity of its ^{13}C spectrum.

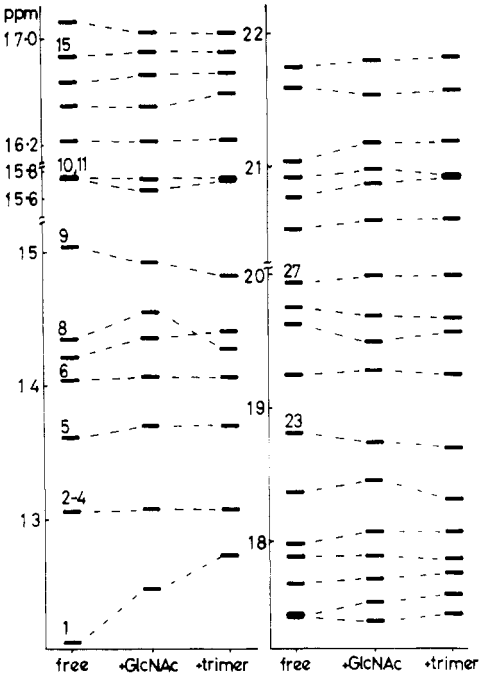

FIGURE 4: Chemical shifts of methyl carbon resonances of lysozyme (6 mM in D_2O) in the absence and presence of either Glc-N-Ac (100 mM) or $(Glc\text{-}N\text{-}Ac)_3$ (12.5 mM), pD 5.0, 40 °C.

Glc-N-Ac and $(Glc\text{-}N\text{-}Ac)_3$ Binding. Glc-N-Ac (2-acetamido-2-deoxy-β-D-glucopyranoside) and its trimer both act as competitive inhibitors of lysozyme. Their primary binding sites are largely known; that of Glc-N-Ac depends slightly on the anomeric form of the amido sugar. In the present study, Glc-N-Ac was present in 16-fold excess at anomeric equilibrium to ensure essentially complete binding at both the pD values studied, viz., 3.2 and 5.0 $(Glc\text{-}N\text{-}Ac)_3$ was in 2-fold excess at pD 5.0, also ensuring complete binding, but it could not be fully bound at pD 3.2.

The binding of either ligand, particularly at pD 5.0, causes detectable shifts (δ >0.03) in the majority of methyl resonances. This contrasts with the nonprotonated aromatic resonances (Allerhand et al., 1977), where only a few are affected. Specifically, 13 out of 30 resolved methyl resonances shift by $\delta \geq 0.15$, whereas only 4 out of 22 aromatic resonances do so, on binding of the monomer. The contrast underlines the extent to which the methyl groups in lysozyme are concentrated in the active site cleft and involved in the flexibility of that region. (See the preceding paper for a similar observation on ribonuclease A.) Figure 4 shows the measured methyl shifts at pD 5.0.

The shift perturbations shown in Figure 4 are so extensive as to inhibit detailed analysis. But some striking features emerge. Peaks 1, 8, 9, and 23 shift substantially, with the shifts of peaks 1 and 9 being approximately doubled on going from Glc-N-Ac to $(Glc\text{-}N\text{-}Ac)_3$. They are assigned elsewhere to Ile-98 C^{γ_2} and Ile-58 C^{δ} or C^{γ_2}, which both lie in the main binding site. A similar increased effect of $(Glc\text{-}N\text{-}Ac)_3$ was observed by Allerhand et al. (1977) for Trp-63 and -111 and Tyr-20 or -23.

Also, there is only one resonance (13/14) that appears to be unperturbed by the binding of Glc-N-Ac but perturbed by the trimer. The second and third residues of the trimer are believed, from model-building studies, to bind at sites that have no methyl groups within 600 pm of the ligand. The only likely candidate for perturbation is Leu-75 C^{δ}, which could be affected via Trp-62 upon occupation of site B (Perkins et al., 1981a,b). Resonance 13 has already been assigned to Met-12

C^{ϵ}. We therefore tentatively assign resonance 14 to Leu-75 C^{δ}. Our experimental observations are consistent with few direct contacts between methyl groups and the ligand but with significant conformational changes on binding, especially with $(Glc\text{-}N\text{-}Ac)_3$.

Perkins et al. (1981a) have shown that at least part of Ile-58 is within 400 pm of the O(7) atom of Glc-N-Ac. Thus, the perturbation of peak 9 by ligand binding supports its assignment to Ile-58 methyl. Perkins & Dwek (1980) have also shown that the methyl groups of Ile-98, Met-105, and Leu-56 are unusual in being under the influence of several tryptophan ring currents. In particular, they show that any movement of Trp-108 should affect Ile-98 C^{δ_2}, Met-105 C^{ϵ}, and Leu-56 C^{δ}. Resonances 1 and 6 have already been assigned above to Ile-98 C^{γ_2} and C^{δ}, respectively. The present marked shift of peak 1 upon binding relative to peak 6 implies that it should be assigned to the C^{δ_2} resonance and also that Trp-108 indeed moves upon Glc-N-Ac binding, as found in the crystal. We have also observed a significant shift in the Trp-108 C^{ϵ_2} resonance to support this. Finally, Met-105 C^{ϵ} was identified as peak 8 from selective decoupling and I_2 oxidation experiments and Leu-56 C^{δ} from decoupling plus Pr^{3+} binding as peak 23. Both these peaks change substantially in shift upon Glc-N-Ac binding, in support of the above hypothesis.

Howarth & Lian (1983) show that Glc-N-Ac markedly reduces the thermal dependence of δ_C arising from methyl groups lying near the active site cleft of lysozyme. We have proposed that binding of the ligand removes one or more alternative conformations accessible to the cleft region at higher temperatures. In further support of our hypothesis, we note that almost all the free-protein shifts upon increasing temperature are in the opposite direction to those observed on binding Glc-N-Ac. Also, they generally tend toward the value expected for a random-coil peptide, although this value is never reached. This implies that the high-temperature native conformation involves less specific ring orientations and is also probably more penetrated by solvent. But, it is still certainly not random coil, both because the denaturation transition is slow and also because many resonances (e.g., Met-105 C^{ϵ}) do not show any thermal shift at all toward either their "peptide" value (Howarth, 1978) or their denatured value, and indeed, some (e.g., Ile-88 C^{γ_2}, Trp-63 C^{ϵ_2}) move in the opposite direction. So, the high-temperature native conformation is a relatively local one, mainly involving atoms in or near the active site cleft.

In contrast, the reversible two-state transition to the thermally denatured state of lysozyme at pD 3.2 and 5.0 involves shifts of all resonances. The denatured state appears to be hydrophobically structured in the same way as described above for urea denaturation and for ribonuclease A. The transition is slow on the NMR timescale, and the denatured spectrum is invariant with temperature or addition of further urea. The ^{13}C spectrum at 80 °C in dimethyl sulfoxide shows that in the aromatic region the shift heterogeneity of all but the C^{γ} resonances is essentially removed, although structuring is still evident in the aliphatic region, particularly with the hydrophilic residues. This supports the contention in the preceding paper that the aqueous denatured structure arises from hydrophobic clustering, whereas ionic forces probably dominate in the nonaqueous solvent.

Conclusions

The binding of amido sugars to lysozyme affects a substantial fraction of atoms in the protein and reduces their conformational freedom markedly. Other chemical perturbations to the protein have less extensive consequences, and some regions of the molecule are relatively inflexible. Indeed, many of the spectroscopic signatures of molecular flexibility such as line broadenings and unusual thermal shifts seem to arise from only a few localized conformational alternatives.

Also, neither urea nor heat seems to affect the native protein to any great extent. There is little or no evidence to support the naive idea of major progressive loosening of the native protein as the denaturation equilibrium is approached. However, the denaturant could well affect the more open denatured state. Using the simple idea of urea in water as a "structure maker", one might propose that, via the solvent, it stabilizes the hydrophobic clusters that are shown above to be present in the denatured protein. This would explain why the structuring of the denatured state, as revealed by the chemical shifts, is unaffected by the addition of further urea, above the minimum necessary for denaturation.

The chemical shifts of the denatured protein are similarly insensitive to temperature. This fact, in combination with the arguments made above against major conformational changes upon heating the native protein, suggests that the denaturing effect of heat may also be mainly due to stabilization of the hydrophobic clusters that dominate the denatured state. Such stabilization is predicted, at least up to 60 °C, by the general theory of hydrophobic bonding (Scheraga, 1963).

Acknowledgments

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Registry No. Pr, 7440-10-0; I_2, 7553-56-2; NBS, 128-08-5; Glc-N-Ac, 7512-17-6; $(Glc\text{-}N\text{-}Ac)_3$, 31175-95-8; lysozyme, 9001-63-2.

References

Allerhand, A., Norton, R. S., & Childers, R. F. (1977) *J. Biol. Chem. 252*, 1786–1794.

Artymiuck, P. J., Blake, C. C. F., Grace, D. E. P., Oakley, S. J., Phillips, D. C., & Sternberg, M. J. E. (1979) *Nature (London) 280*, 563–568.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, Ser. B 167*, 365–377.

Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1975) *Proc. R. Soc. London, Ser. A 345*, 41–59.

Delepierre, M., Dobson, C. M., & Poulsen, F. M. (1982) *Biochemistry 21*, 4756–4761.

Dobson, C. M. (1975) D. Phil. Thesis, University of Oxford, U.K.

Doddrell, D. M., Pegg, D. T., & Bendall, M. R. (1982) *J. Magn. Reson. 48*, 323–327.

Howarth, O. W. (1978) *Prog. Nucl. Magn. Reson. Spectrosc. 12*, 1–40.

Howarth, O. W., & Lian, L. Y. (1983) *J. Chem. Soc., Chem. Commun.*, 434.

Howarth, O. W., & Lian, L. Y. (1984) *Biochemistry* (preceding paper in this issue).

Jori, G., Galiazzo, G., & Scaffone, E. (1969) *Biochemistry 8*, 2868–2875.

Norton, R. S., & Allerhand, A. (1976a) *J. Biol. Chem. 251*, 6522–6528.

Norton, R. S., & Allerhand, A. (1976b) *Biochemistry 15*, 3438–3445.

Perkins, S. J., & Dwek, R. A. (1980) *Biochemistry 19*, 245–258.

Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A.

(1981a) *Biochem. J. 193*, 553–572.
Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1981b) *Biochem. J. 193*, 573–588.
Poulsen, F. M., Hoch, J. C., & Dobson, C. M. (1980) *Biochemistry 19*, 2597–2607.
Scheraga, H. A. (1963) *Proteins*, 545.
Shindo, H., & Cohen, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1979–1983.

^{1}H NMR Spectroscopy of *Paracoccus denitrificans* Cytochrome *c*-550†

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ABSTRACT: The ^{1}H NMR spectra of ferri- and ferrocytochrome *c*-550 from *Paracoccus denitrificans* (ATCC 13543) have been investigated at 300 MHz. The ferricytochrome *c*-550 shows hyperfine-shifted heme methyl resonances at 29.90, 29.10, 16.70, and 12.95 ppm and a ligand methionyl methyl resonance at −15.80 ppm (pH 8 and 23 °C). Four pH-linked structural transitions were detected in spectra taken as a function of pH. The transitions have been interpreted as loss of the histidine heme ligand ($pK \leq 3$), ionization of a buried heme propionate ($pK = 6.3 \pm 0.2$), displacement of the methionine heme ligand by a lysyl amino group ($pK \simeq 10.5$), and loss of the lysyl ligand ($pK \geq 11.3$). The temperature behavior of hyperfine-shifted resonances was determined. Two heme methyl resonances (at 16.70 and 12.95 ppm) showed downfield hyperfine shifts with increasing temperature. The cyanoferricytochrome had methyl resonances at 23.3, 20.1, and 19.4 ppm. NMR spectroscopy did not detect the formation of a complex with azide. The second-order rate constant for electron transfer between ferric and ferrous forms was determined to be 1.6×10^4 M^{-1} s^{-1}. Heme proton resonances were assigned in both oxidation states by cross-saturation and nuclear Overhauser enhancement experiments. Spin-coupling patterns in the aromatic region of the ferrocytochrome spectrum were investigated.

Cytochromes *c* are found widely distributed in all eukaryotic species as well as in most prokaryotic species. As a general trend, these cytochromes *c* show species specificity for electron-transfer reactions with coupled proteins. Notable exceptions have been electron-transport proteins from *Paracoccus denitrificans*, which have been shown to cross-react with components of the mitochondrial electron-transport chain (Smith et al., 1976; Timkovich et al., 1982). On the basis of these and other metabolic comparisons, it has been proposed that *Paracoccus* may be closely related to the prokaryote that evolved into the mitochondrion of higher organisms (John & Whatley, 1975). The respiratory electron-transport chain of *Paracoccus* has been intensively studied (John & Whatley, 1977), especially the properties of the main soluble *c*-type cytochrome, termed cytochrome *c*-550. Its crystal structure has been determined (Timkovich & Dickerson, 1976) as has its amino acid sequence (Timkovich et al., 1976; Ambler et al., 1981). Detailed investigations have been made of the kinetic properties and immunological cross-reactions (Kuo et al., 1983) of this protein.

The purpose of this paper is to extend the structural characterization of this cytochrome by ^{1}H NMR spectroscopy. ^{1}H NMR is a valuable technique to monitor the electronic environment in the vicinity of the heme, because the hyperfine chemical shifts induced by the iron paramagnetism of the ferric state are highly sensitive to distances and orientations (the pseudocontact interaction) and electron-density distribution (the contact interaction). In this regard, it compliments X-ray crystallography, which is insensitive to the distribution of outer shell electrons. In the ferrous state, high-field NMR detects narrow lines of heme and amino acid side-chain protons. Precise chemical shifts, line widths, and, in some cases, spin-coupling patterns provide information on conformation. For example, a detailed NMR comparison of horse and tuna cytochromes *c* (Moore & Williams, 1980b) has elucidated fine structural differences in the solution state not readily apparent in the respective crystal structures. Prokaryotic cytochromes *c* show greater structural diversity among themselves and in comparison to eukaryotic cytochromes than is found among just eukaryotic cytochromes considered as a subclass. As additional prokaryotic cytochromes are studied by ^{1}H NMR, a library of spectra and assignments is growing. This expanding library will create new avenues for the comparison of protein structures.

Materials and Methods

Paracoccus denitrificans (ATCC 13543) was cultured and cytochrome *c*-550 was isolated as described by Scholes et al. (1971). A final chromatography step on hydroxyapatite was included in the purification (Ambler et al., 1981). The purity ratio (absorbance at the Soret maximum to absorbance at 280 nm for the oxidized form) of the final material was 5.2. This is equivalent to the value that may be calculated from the spectrum of pure cytochrome given in Scholes et al. (1971). In some experiments, material with a purity ratio of 4 was employed. Spectra of this material did not differ in the critical spectral regions of interest from fully purified cytochrome. Samples for NMR were dialyzed vs. 50 mM ammonium bicarbonate, pH 7.8, lyophilized, and redissolved in deuterated buffer. This solvent was 99.8% deuterium oxide, 10 mM potassium phosphate, and 100 mM sodium chloride, adjusted to the appropriate pH. Values labeled pH* represent the direct reading of a glass combination electrode in deuterium oxide after the electrode had been calibrated in protic reference solutions. Adjustments of pH for titration studies were made by the addition of aliquots of ^{2}HCl or NaO^{2}H. Potassium cyanide and sodium azide were added to protein samples from concentrated stock solutions in ^2H_2O adjusted to the same pH* as the protein. Cytochrome reduction was accomplished by the addition of a minimum amount of solid sodium dithionite.

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