Volume 213, number 2, 391-395 FEB 04526 March 1987

NMR study of the interaction between cytochrome b_5 and cytochrome c

Observation of a ternary complex formed by the two proteins and $[Cr(en)_3]^{3+}$

R. Timothy Hartshorn°, A. Grant Mauk*, Marcia R. Mauk* and Geoffrey R. Moore+

*Inorganic Chemistry Laboratory, South Parks Road, Oxford OX13 QR, England, *Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada* and *+School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, England*

Received 5 January 1987

The interaction between horse cytochrome c and the tryptic fragment of bovine liver microsomal cytochrome b_5 in the absence and presence of $[Cr(ethylenediamine)_3]Cl_3$ was studied by ^1H NMR spectroscopy. The protein-protein interaction region on cytochrome b_5 was found to be different from the $[Cr(en)_3]^{3+}$-binding region. The solvent-exposed propionate-bearing edge of the haem of cytochrome b_5 is accessible to $[Cr(en)_3]^{3+}$ in the interprotein complex.

^1H-NMR; Cytochrome c; Cytochrome b_5; Protein complex

1. INTRODUCTION

Electron transfer reactions between two metalloproteins generally require the formation of an intermolecular complex of the two proteins in which electron transfer occurs. Several such complexes involving mitochondrial cytochrome c have been studied, including those with the structurally defined proteins cytochrome b_5 [1] and cytochrome c peroxidase [2]. Although X-ray diffraction structures of such protein-protein complexes have not been described, hypothetical structures have been proposed based on the use of computer graphics to align the three-dimensional structures [3,4] of the two partner proteins. Characterisation of the structural features of these two complexes in solution provides a useful means of assessing the validity of computer modelling of such complexes.

Various lines of experimental work have established that cytochrome b_5 and c do form a stable bimolecular complex in solution [5], and chemical modification [6,7] and NMR [8] studies of cytochrome c indicate that the Salemme model is approximately correct in identifying the region of cytochrome c that forms the cytochrome b_5 interaction domain even though the specific roles of particular residues have not been fully defined.

Recent studies of the role of a cytochrome b_5 haem propionate group in complex formation with cytochrome c have led to the proposal that at least one alternative docking orientation possesses electrostatic stability equal to that of the Salemme

Correspondence address: A.G. Mauk, Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada or G.R. Moore, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, England

° Present address: Department of Inorganic Chemistry, University of Newcastle, Newcastle, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

complex under specific solution conditions [9]. The present paper reports the ^1H NMR spectroscopy examination of the cytochrome b_5-c interaction using the paramagnetic-reagent-competition approach described in [8]. Our results are consistent with a model in which binding $[Cr(en)_3]^{3+}$ to an exposed haem propionate of complexed cytochrome b_5 occurs. The apparent conflict between this model and the Salemme model is discussed.

2. MATERIALS AND METHODS

The tryptic fragment of bovine liver microsomal cytochrome b_5 and its derivative with ferriprotoporphyrin IX dimethyl ester were prepared as in [10,11]. ^2H_2O solutions of these proteins for NMR were prepared by exchanging the solvent with an Amicon diafiltration cell equipped with a YM-5 membrane. pH adjustments were done with ^2HCl and NaO^2H, and the quoted values designated pH* are the direct meter readings uncorrected for the small isotope effect [12].

Horse-heart cytochrome c (type VI) was obtained from Sigma (Poole, England) and prepared for NMR as in [13]. $[Cr(en)_3]Cl_3$ (en, ethylenediamine) was obtained from Ventron-Alfa (MA, USA).

NMR spectra were recorded with a Bruker 300 MHz spectrometer. Paramagnetic difference spectra (PDS) were obtained as described [14]. 1,4-Dioxan was used as an internal standard, but all chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

3. RESULTS AND DISCUSSION

3.1. $[Cr(en)_3]^{3+}$ binding to ferricytochrome b_5

Assignments of haem resonances of native ferricytochrome b_5 have been described [15,16], and relevant assignments are given in table 1 along with corresponding assignments of the dimethyl ester (DME) derivative. The large perturbations of some of the haem methyl chemical shifts on esterification result from alteration in the distribution of the unpaired electron spin of the haem.

On addition of $[Cr(en)_3]^{3+}$ to ferricytochrome b_5 at pH* 7.0, some of the haem resonances specifically broaden, in particular resonances of haem methyl 5 and haem propionates 6 and 7 are affected (fig.1). A number of resonances in the region 0–10 ppm are also broadened (not shown), but these have not yet been assigned.

Table 1

Resonance assignments for ferricytochrome b_5 and DME-ferricytochrome b_5

Proton	δ (ppm)	
	Native b_5 (pH* 7.0, 25°C) [15,16]	DME-b_5 (pH* 6.8, 25°C) [this work]
Haem methyl-1	11.65	11.35
Haem methyl-3	14.30	16.50
Haem methyl-5	21.75	17.55
Haem vinyl 2 αCH	27.30	26.15
Haem propionate 6 αCH_2	15.80	n.a.
Haem propionate 7 αCH	19.00	n.a.

n.a., not assigned. The DME-b_5 assignments were obtained for nuclear Overhauser enhancements

The resonance at 27.3 ppm (fig.1) is shifted upon addition of $[Cr(en)_3]^{3+}$. This is an unusual effect to be caused by a relaxation enhancement reagent, and it arises because the chemical shift of the resonance is determined largely by the distribution of unpaired electron spin in the haem and this is affected by the cation binding. The proton is part of haem vinyl-2 and this is too far from the bound cation to be significantly relaxed by it. The chemical shift variation is consistent with an apparent association constant of 380 M^{-1}; the ionic strength was not controlled in this experiment, and it varied over the approximate range 0.02–0.05 M. This apparent association constant is in reasonable agreement with the value of 309 M^{-1} at an ionic strength of 0.1 M reported by Chapman et al. [17].

Addition of $[Cr(en)_3]^{3+}$ to DME-ferricytochrome b_5 at pH* 6.8 produced far weaker effects than for the native protein, and even at 120 mol% $[Cr(en)_3]^{3+}$ haem methyl 5 was only slightly affected (fig.2). This finding indicates a marked reduction in the binding of $[Cr(en)_3]^{3+}$ to the modified proteins compared to the native cytochrome.

The broadening effects accompanying $[Cr(en)_3]^{3+}$ binding to native ferricytochrome b_5 are similar to those observed by McLachlan et al.

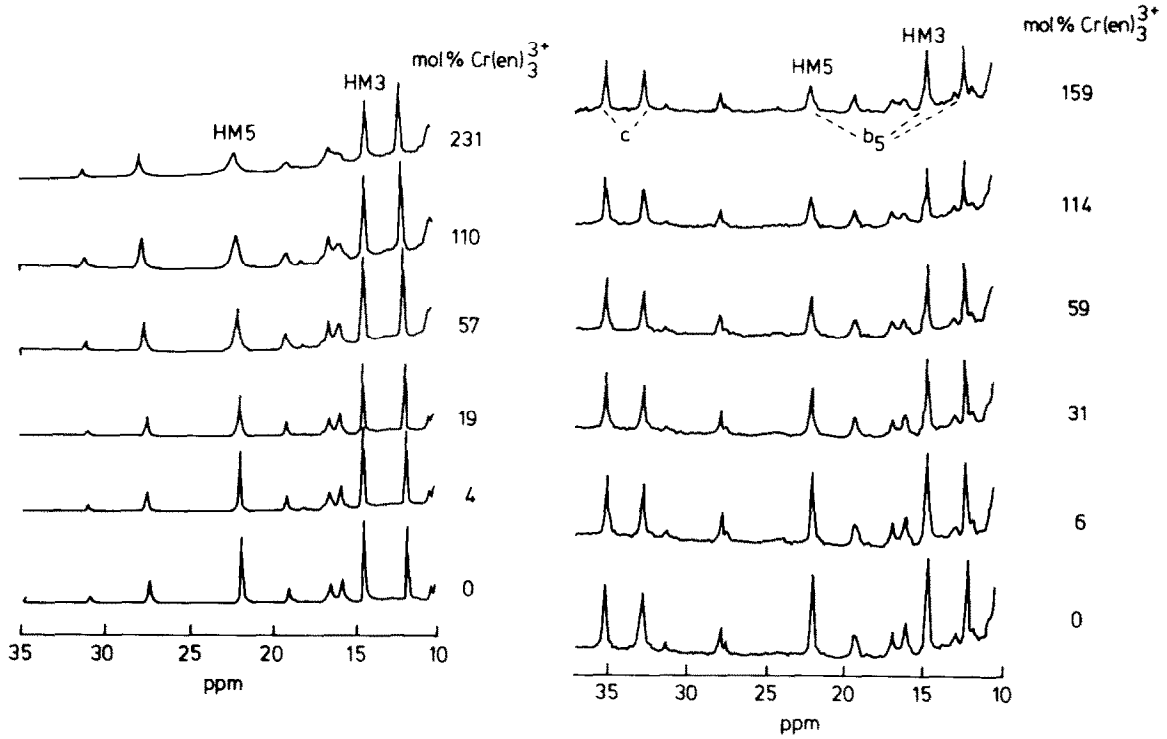

Fig.1. Regions of the ^1H NMR spectra of ferricytochrome b_5 (left) and a ferricytochrome b_5-c mixture (right) in ^2H_2O at pH* 7.0 and at 25°C, with varying concentrations of $[Cr(en)_3]Cl_3$. Cytochrome concentrations were 3×10^{-3} M. Haem methyl resonances of cytochrome c and cytochrome b_5 are indicated in the spectra of the mixture. HM3 and HM5, resonances of haem methyls 3 and 5 of ferricytochrome b_5.

[16] for Gd^{3+} binding at pH* 6.4, and they indicate the existence of a cation-binding site close to the haem. McLachlan et al. [16] suggest that haem propionate 6 is one of the key groups in this site. The observation that esterification of the haem propionates drastically reduces the association of $[Cr(en)_3]^{3+}$ to cytochrome b_5 is consistent with a role for the haem propionates in binding small cations. Matthews et al. [4] have also suggested that haem propionate 6 binds cations in ferricytochrome b_5.

3.2. *Competition between ferricytochrome* c *and* $[Cr(en)_3]^{3+}$ *for ferricytochrome* b_5

The NMR spectrum of the haem resonances of ferricytochrome b_5 in the presence of ferricytochrome c is changed little compared to that in the absence of cytochrome c (fig.1). Some resonances of cytochrome c are markedly shifted by the presence of cytochrome b_5 however, and these have been discussed previously [8]. These shifts coupled with the general broadening of the main protein absorption envelope between 0 and 10 ppm (not shown) are indicative of complex formation and their continuing presence throughout the $[Cr(en)_3]^{3+}$ titration of the cytochrome mixture shows that protein-protein complex formation occurs in the presence of the Cr^{3+} complex.

Addition of $[Cr(en)_3]^{3+}$ to the mixture caused perturbations similar to the haem resonances (figs 1,2) and amino acid resonances (not shown) of ferricytochrome b_5 as for ferricytochrome b_5 in the absence of cytochrome c. This shows that $[Cr(en)_3]^{3+}$ is able to bind close to the haem of cytochrome b_5 in the cytochrome b_5-c complex and that the binding of cytochrome c to cytochrome b_5 does not significantly inhibit the binding of $[Cr(en)_3]^{3+}$.

3.3. *The cytochrome* b_5-c *complex and the NMR competition method*

PDS are not needed to resolve the haem

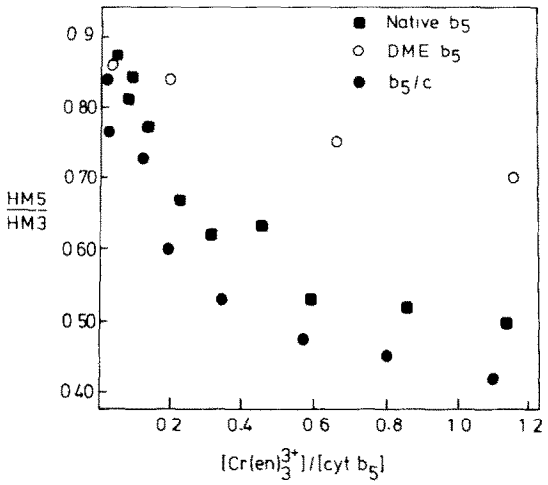

Fig.2. Plots of the ratio of peak heights for haem methyls 5 and 3 of ferricytochrome b_5 as a function of the ratio of $[Cr(en)_3]^{3+}$: cytochrome b_5. The peak height ratio is a normalising procedure to take account of changes in linewidth not associated with the $[Cr(en)_3]^{3+}$ (cf. the two control spectra in fig.1). The procedure is valid because haem methyl 3 is not greatly affected by $[Cr(en)_3]^{3+}$.

resonances of cytochrome b_5, and although PDS were obtained for the main amino acid regions, these are not at present helpful in locating cation-binding sites. However, the haem resonance data indicate that $[Cr(en)_3]^{3+}$ binds close to the haem of cytochrome b_5, and probably to at least one of its haem propionates, in both the absence and presence of cytochrome c. There are a number of situations that could give rise to this result.

(i) $[Cr(en)_3]^{3+}$ could dissociate the cytochrome complex and bind to free cytochrome b_5. The constant broadening and shift of cytochrome c resonances argue against this in the present case.

(ii) Cytochrome c could bind to cytochrome b_5 at the latter's haem edge but the addition of $[Cr(en)_3]^{3+}$ causes a shift in the position of the bound cytochrome c so that although an inter-protein complex is maintained the cytochrome b_5 haem edge is free to bind $[Cr(en)_3]^{3+}$. That is, binding of $[Cr(en)_3]^{3+}$ induces formation of an alternative cytochrome c:b_5 docking alignment.

(iii) Cytochrome c and $[Cr(en)_3]^{3+}$ may bind to different regions of the cytochrome b_5 surface. Cytochrome b_5 is a highly anionic protein (net charge -9 at pH 7), and its cation-binding surface is large enough [9] to bind both cytochrome c and $[Cr(en)_3]^{3+}$ simultaneously.

At present it is not clear whether scheme (ii) or (iii) best describes the ternary complex.

3.4. *Comparison with previous studies*

The central comparison to be made is between the present work and Salemme's model [1]. That model proposed that the haem edge of cytochrome b_5 was buried within the cytochrome b_5-c complex with haem propionate 7 forming a salt bridge with Lys 79 of cytochrome c. Recently, measurements of the stability of complex formation between DME-cytochrome b_5 and cytochrome c combined with electrostatics calculations have led to the conclusion [9] that blocking of the cytochrome b_5 haem propionate groups (as methyl esters) can result in an alternative docking geometry for complex formation that is electrostatically isoenergetic with the Salemme complex at pH 7. For the native proteins, parallel calculations verified that the Salemme complex is the electrostatically favoured arrangement at neutrality. Significantly, the esterified haem propionates of cytochrome b_5 are fully accessible to solvent in the new complex which leads to the proposal that the effect of $[Cr(en)_3]^{3+}$ binding to cytochrome b_5 interacting with cytochrome c is equivalent to the effect of propionate group esterification in that formation of the alternative complex is favoured [this is scheme (ii)]. However, the NMR results are also consistent with a model in which the cytochrome b_5 alignment proposed by Salemme is incorrect. Whichever of the two proposals is correct, one point is clear. The interaction between the different charged species is not determined solely by their net charge because in that case $[Cr(en)_3]^{3+}$ and cytochrome b_5 should compete strongly with each other. Finally, we stress that the structure of the complex formed between these two proteins in solution may not be static [8,9], as is sometimes inferred from computer-modelling studies. Clearly, a range of protein alignments that result in complexes of comparable thermodynamic stability is

likely [9] and the effect of $[Cr(en)_3]^{3+}$ may be to change the relative stabilities of different protein orientations so that a range of different alignments is produced.

ACKNOWLEDGEMENTS

This research was supported by the UK Science and Engineering Research Council (SERC) and the US National Institutes of Health (through grant GM-28834 to A.G.M.). G.R.M. thanks the SERC for the award of an Advanced Fellowship.

REFERENCES

[1] Salemme, F.R. (1976) J. Mol. Biol. 102, 563–568.
[2] Poulos, T.L. and Kraut, J. (1980) J. Biol. Chem. 255, 10322–10330.
[3] Takano, T. and Dickerson, R.E. (1981) J. Mol. Biol. 153, 79–115.
[4] Matthews, F.S., Czerwinski, E.W. and Argos, P. (1979) in: The Porphyrins (Dolphin, D. ed.) vol.7, pp.107–147, Academic Press, New York.
[5] Mauk, M.R., Reid, L.S. and Mauk, A.G. (1982) Biochemistry 21, 1843–1846.
[6] Ng, S., Smith, M.B., Smith, H.T. and Millett, F. (1977) Biochemistry 16, 4975–4978.
[7] Stonehuerner, J., Williams, J.B. and Millett, F. (1980) Biochemistry 18, 5422–5427.
[8] Eley, C.G.S. and Moore, G.R. (1983) Biochem. J. 215, 11–21.
[9] Mauk, M.R., Mauk, A.G., Welser, P.C. and Matthew, J.B. (1986) Biochemistry, in press.
[10] Reid, L.S. and Mauk, A.G. (1982) J. Am. Chem. Soc. 104, 841–845.
[11] Reid, L.S., Mauk, M.R. and Mauk, A.G. (1984) 106, 2182–2185.
[12] Kalinichenko, P. (1976) Stud. Biophys. 58, 235–240.
[13] Eley, C.G.S., Moore, G.R., Williams, R.J.P., Neupert, W., Boon, P.J., Brinkhof, H.H.K., Nivard, R.J.F and Tesser, G.I. (1982) Biochem. J. 205, 153–165.
[14] Eley, C.G.S., Moore, G.R., Williams, G. and Williams, R.J.P. (1982) Eur. J. Biochem. 124, 295–303.
[15] Keller, R.M. and Wüthrich, K. (1980) Biochim. Biophys. Acta 621, 204–217.
[16] McLachlan, S.J., LaMar, G.N. and Sletten, E. (1986) J. Biol. Chem. 108, 1285–1291.
[17] Chapman, S.K., Davies, D.M., Vuik, C.P.J. and Sykes, A.G. (1983) J. Chem. Soc. Chem. Commun. 868–869.