NUCLEAR MAGNETIC RESONANCE SPECTRA OF HUMAN AND BOVINE SUPEROXIDE DISMUTASES

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Received 9 March 1973

1. Introduction

Human and bovine erythrocyte superoxide dismutases have been isolated and shown to be of almost identical chemical composition [1, 2]. Nuclear magnetic relaxation dispersion studies on bovine superoxide dismutase have indicated that a rapidly exchanging water molecule occupies one of the copper ligand sites [3].

In the present paper we wish to describe evidence giving the presence of histidine residues at or close to the copper binding sites of both human and bovine superoxide dismutases. This evidence was obtained from ^{1}H NMR spectra at 270 MHz of human and bovine superoxide dismutases in the native and apo form.

2. Materials and methods

Bovine erythrocyte superoxide dismutase was prepared as already described [1]. The human enzyme was prepared by a modification of the procedure of Bannister et al. [2]. Crude human erythrocyte superoxide dismutase was prepared by ethanol:chloroform (3:1, v/v) fractionation of outdated human red blood cells. The crude protein was purified by three stages of chromatography on QAE-Sephadex (Pharmacia Fine Chemicals AB). Column dimensions were 2.5 × 30 cm for the protein from 4–5 ℓ of packed red blood cells. The column was eluted with starting buffer (0.02 M cacodylate-HCl, pH 6.5), then with 0.02 M cacodylate-HCl buffer at pH 5.5 and finally with 0.05 M cacodylate-HCl at pH 5.5. The enzyme was eluted with the final buffer. The purified protein was dialysed against water and lyophilised. The final preparation was homogeneous by polyacrylamide gel electrophoresis and ultracentrifugation. The yield was about 20 mg of protein per ℓ of packed red blood cells. The human and bovine apo proteins were prepared by the method of Weser et al. [4]. Copper and zinc were determined in the holo and apo proteins by Atomic Absorption Spectroscopy (Perkin Elmer Model 303).

Prior to ^{1}H NMR experiments, protein samples (approx. 10 mg/ml) were incubated in 99.8% deuterium oxide (Norsk Hydro-Electrisk) at room temp. for up to 72 hr and lyophilised in order to deuterate exchangeable protons. ^{1}H NMR spectra were obtained for protein solutions in deuterium oxide using the modified Bruker HFX-90 console and 64 Kgauss superconducting magnet (Oxford Instruments Ltd.) of the Oxford Enzyme Group. All protein solutions contained 0.4 mM TSS (3-(trimethylsilyl)-propane sulphonate) as internal standard.

3. Results and discussion

The 270 MHz ^{1}H NMR spectra of human and bovine apo superoxide dismutases are shown in fig. 1 (A and D). They show features typical of the NMR spectra of relatively small proteins with ring-current shifted methyl resonances at high-field, numerous resolved resonances to lower field and finally the resonances due to aromatic residues at low-field. Perhaps the most striking feature of these spectra is the apparent simplicity of the

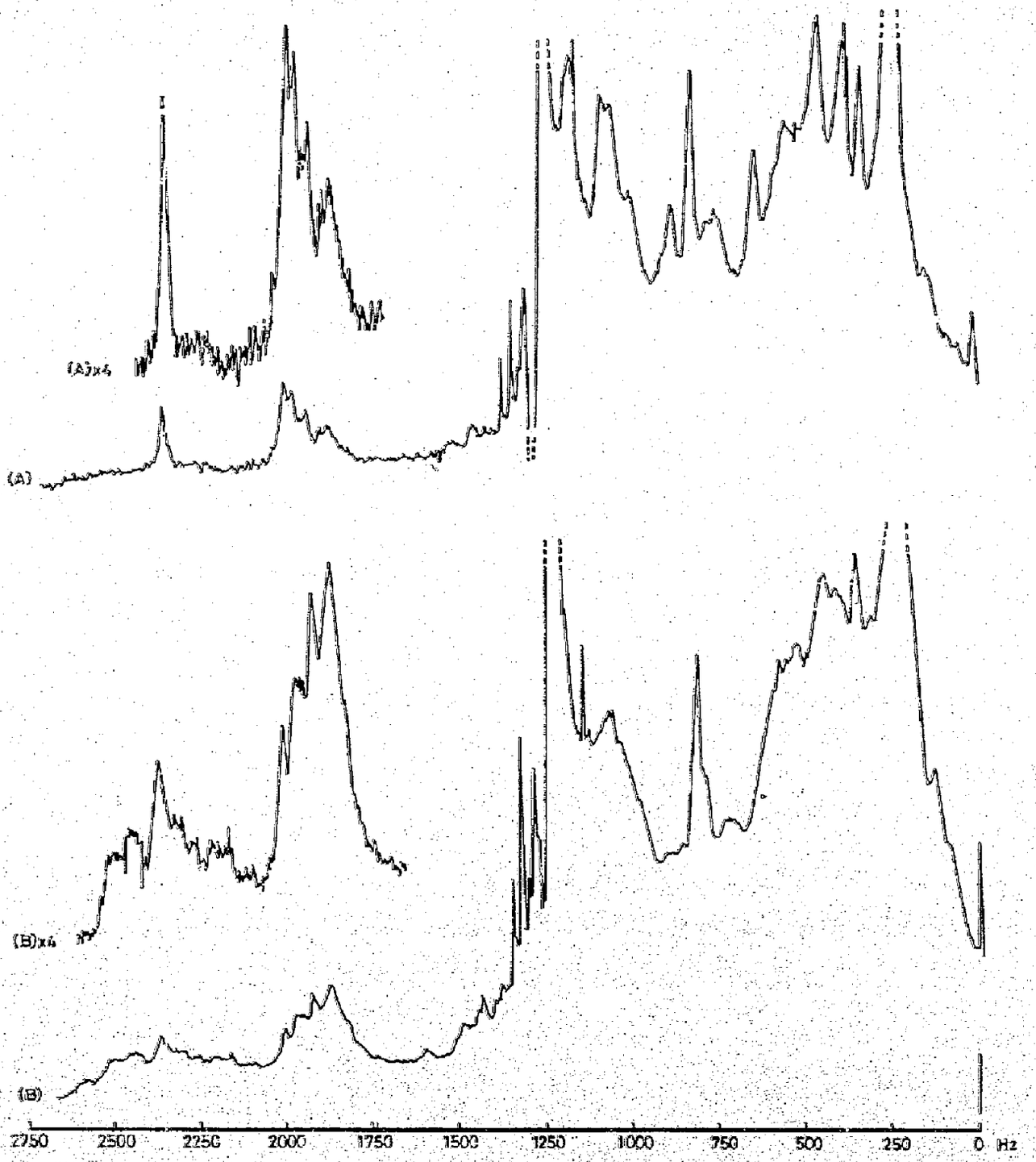

Fig. 1. ^{1}H NMR spectra at 270 MHz of erythrocyte superoxide dismutase in deuterium oxide at pH 5.5 and 40°. (A) Human apo protein; (B) human holoprotein. Shifts are measured with respect to the TSS internal standard.

low-field (aromatic) region (1750–2400 Hz from internal TSS). In particular the peaks at 2366 Hz in the spectra of the human (fig. 1A, peak I) and bovine (fig. 1D, peak II) apo proteins may be assigned as imidazole C-2 resonances [5] and correspond to approx. 6 histidine residues per molecule of protein.

In marked contrast to the apo protein spectra the ^{1}H NMR spectra of the human and bovine native superoxide dismutases (fig. 1B and 1E) show no distinct resonance at 2366 Hz. Consequently sharp resonances at this frequency are a prominent feature of the relevant difference spectra (fig. 1C and 1F). The difference spectra also show sharp resonances between 1920 and 2070 Hz in the aromatic region and throughout the methyl region (700–0 from TSS). Though many of these spectral differences may be due to differences in conformation of superoxide dismutase in the native and apo forms — the major effect is presumably due to the paramagnetic copper(II) which will affect the relaxation times of the proton resonances and this is reflected in the pronounced broadening of the imidazole resonances (peaks I and II) in the native protein.

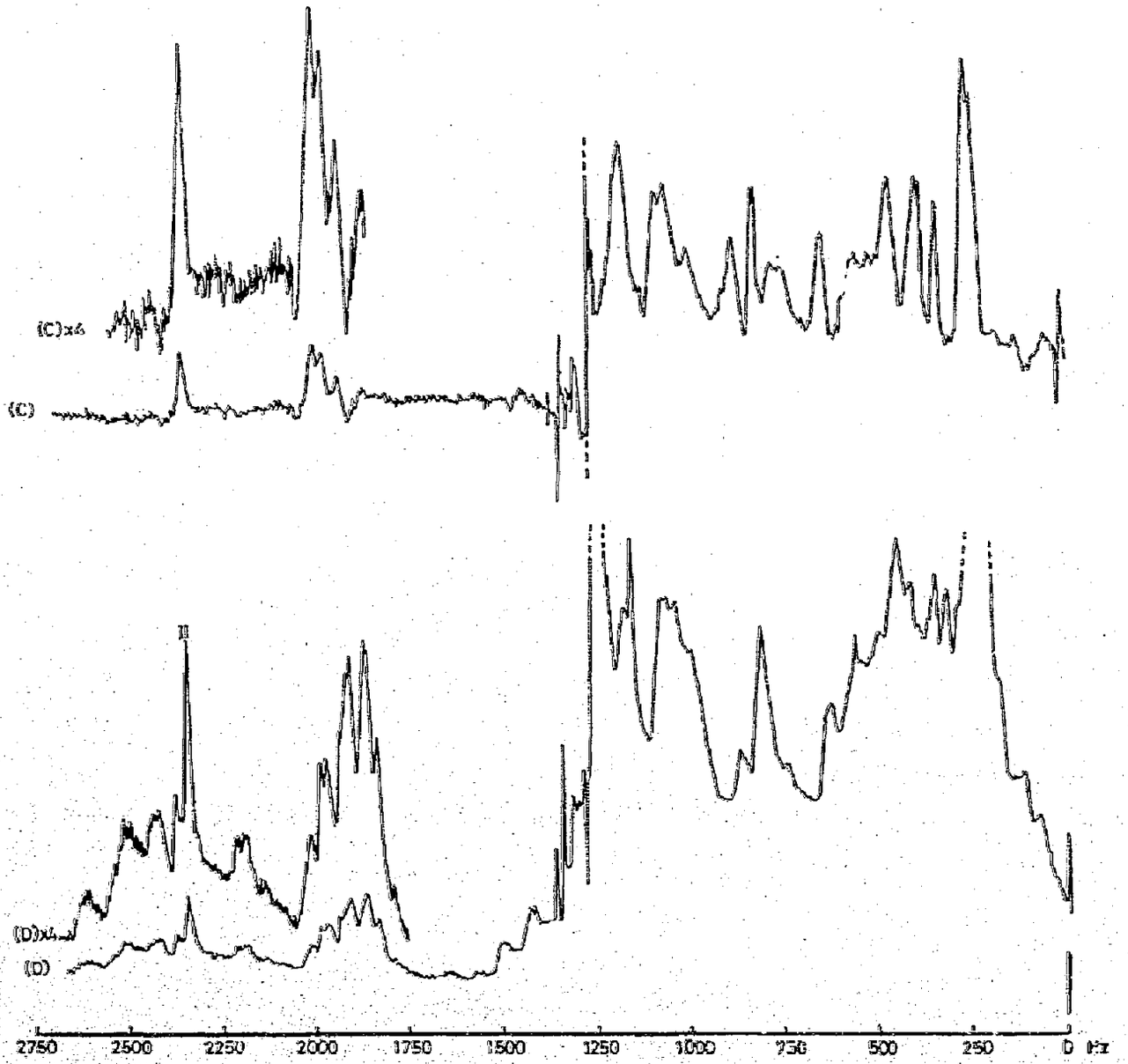

Fig. 1. ^{1}H NMR spectra at 270 MHz of erythrocyte superoxide dismutase in deuterium oxide at pH 5.5 and 40°. (C) difference spectrum (A–B); (D) bovine apo-protein. Shifts are measured with respect to the TSS internal standard.

Reduction with sodium dithionite gives a diamagnetic copper(I) protein which has an NMR spectrum similar to that of the apo protein, suggesting that no major structural change occurs on binding of the copper.

The marked effect on the histidine resonances suggests that they are relatively close to the Cu^{2+} ions. The histidine residues giving rise to these broadened resonances must be of particular interest since at least one of the two copper binding sites of superoxide dismutase has been identified as the catalytic centre of the enzyme [6]. Recent evidence obtained from EPR spectra suggests that each copper(II) ion has three nitrogenous ligands [7, 8]. If these ligands were histidine residues they would give dramatically broadened resonances.

To arrive at more quantitative information on the structure of the protein making use of the copper(II) ions as intrinsic perturbations of the ^1H NMR spectra, a study of systems where the copper(II) is in a "fast exchange" condition has to be made. This is difficult

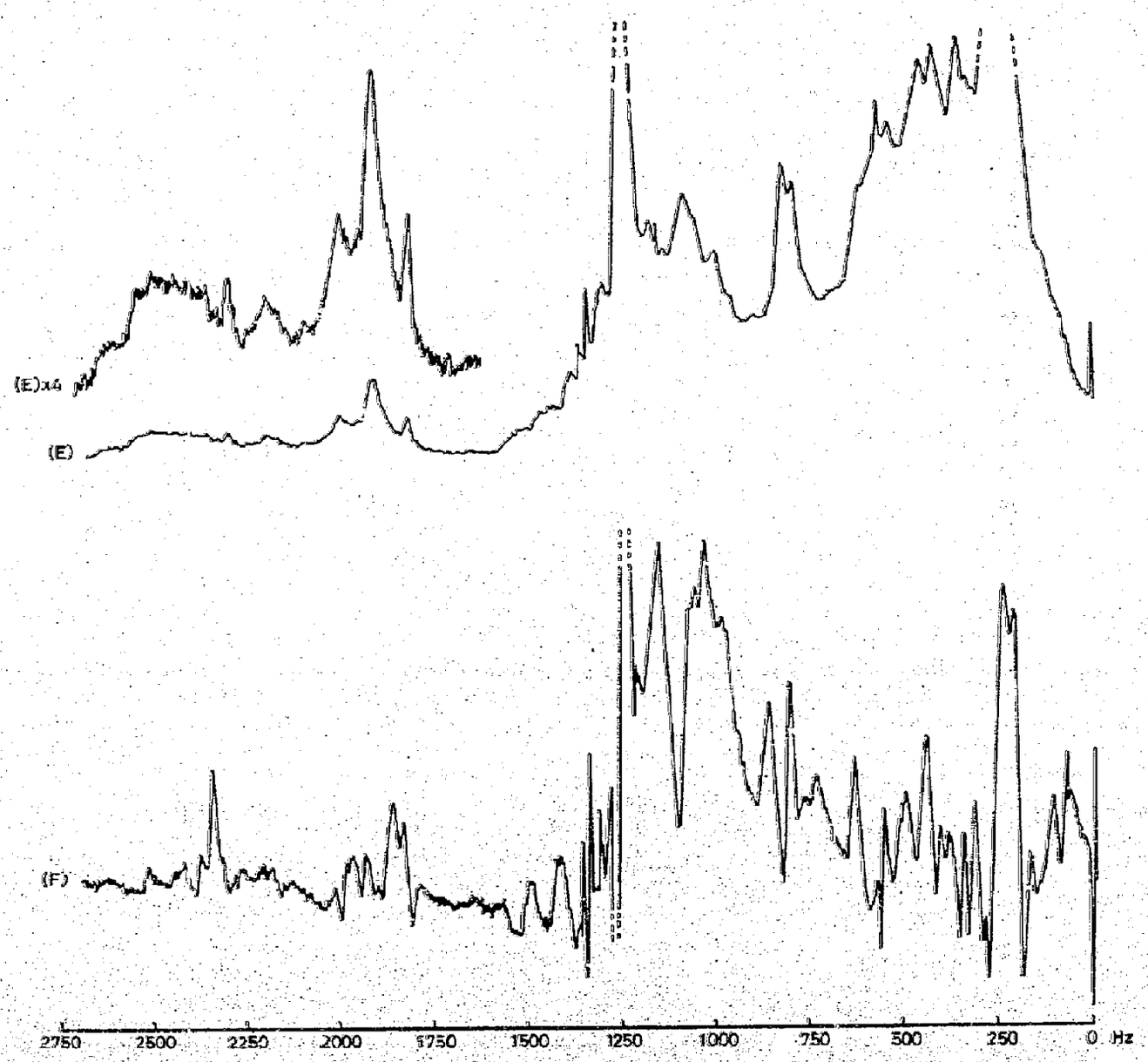

Fig. 1. ^1H NMR spectra at 270 MHz of erythrocyte superoxide dismutase in deuterium oxide at pH 5.5 and 40°. (E) bovine holoprotein and (F) difference spectrum (D–E). Shifts are measured with respect to the TSS internal standard.

to achieve in the sense of chemical exchange, but we can arrive at a fast exchange situation by making use of electron exchange between the protein in its copper(II) and copper(I) forms. The fast electron exchange situation is approached on titration of the holoenzyme with potassium ferrocyanide, which approximates to the "poised-potential" situation [9]. In the NMR spectrum a gradual change is observed from the broadened spectrum of the holoenzyme to the characteristically sharp spectrum of the diamagnetic protein. It may therefore be possible to relate the position of these residues which give rise to assignable resonances to that of the copper(II) ions.

Such techniques, combining the use of difference spectroscopy [10] and the intrinsic probe properties of Cu^{2+} should be applicable to any copper protein which exists in the paramagnetic and diamagnetic states.

Acknowledgements

We thank the SRC (A.M.S. and H.A.O.H.), the Wellcome Trust and the Nuffield Foundation (W.H.B.) for financial support. H.A.O.H. is a member of the Oxford Enzyme Group. J.V.B. thanks Dr. P.J.R. Phizackerley and Mr. J.R.P. O'Brien for much encouragement.

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