A PROTON MAGNETIC RESONANCE STUDY OF THE INTERACTION BETWEEN COPPER AND AZURIN

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1. Introduction

In a previous paper Finazzi Agrò et al. [1] suggested the presence of a tryptophanyl and a cysteinyl residue in the copper binding site of azurin. This is a "blue" protein containing a copper atom per molecule. The blue colour corresponds to a strong absorption at 630 nm and is associated with a characteristic electron spin resonance spectrum [2]. The copper is easily removed from the protein moiety. The apoprotein combines stoichiometrically with copper (II) salts to give a protein which is indistinguishable from the original one. In the present paper we report the modifications of the proton magnetic resonance spectrum of this protein in the presence and in the absence of copper, in an attempt to gain further information concerning the copper site. The results show that High Resolution NMR might represent a very powerful tool also in the investigations of the metal site of metalloproteins.

While this work was in progress, a comparable experimental approach was attempted by Woodworth et al. [3], for the metal binding sites of siderophilin and conalbumin.

2. Materials and methods

All chemicals used were of reagent grade. Deuterium oxide, 99.75% enriched was from Merck A.G., Darmstadt. Azurin was prepared according to Ambler [4] with the modifications described by Finazzi Agrò et al. [1]. The copper-free azurin was prepared according to Yamanaka et al. [5]. All preparations were tested for the copper content by atomic absorption spectroscopy with an Hilger and Watts Atomspek Model H 1170, and for the recombination capacity with the spectrofluorometric method previously described [1]. The apoprotein was freeze-dried, dissolved in D_2O and freeze-dried again. The procedure was repeated three times, to ensure maximal H_2O removal from the protein. $CuCl_2 .2H_2O$ used for recombination studies was heated in an oven at 80° until disappearance of the blue color, then was dissolved in D_2O; the procedure was repeated several times.

Proton NMR spectra were taken with a Varian HA-100 Spectrometer, at a working frequency of 100 MHz and at room temperature (23°). All spectra were run using HMDS (hexamethyldisiloxane) as external reference contained in a capillary concentrical to the standard 5 mm absorbance sample cells. No correction for different magnetic susceptibility of the samples was made.

3. Results

The proton magnetic resonance spectra of native, copper-free partially and fully reconstituted azurin are shown in fig. 1. Figs. 2 and 3 show more detailed spectra of the 1 to 4 and 6 to 10 ppm regions of apoprotein and reconstituted azurin. Significant differences are evident in the absence of the metal: (I) a

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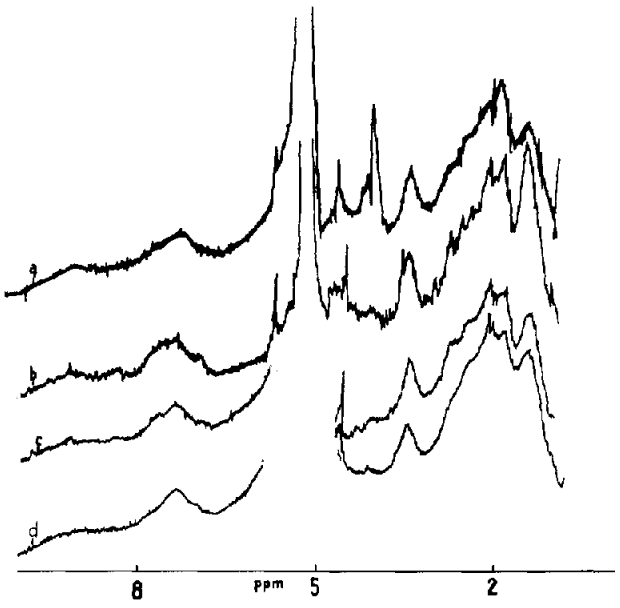

Fig. 1. Proton magnetic resonance spectra at 100 MHz of 20 mM azurin. Spectrum a: native protein; spectrum b: copper-free protein; spectrum c: after addition of 10 mM $CuCl_2$ to the sample of spectrum b; spectrum d: after addition of 20 mM $CuCl_2$ to the sample of spectrum b.

peak appears at 7.7 ppm in the copper-free protein; (II) a sharp and isolated resonance is detected in the apoazurin spectrum at 3 ppm and disappears in the copper containing samples; (III) the large absorption at higher field increases when copper is removed; (IV) a broadening of many peaks is brought about by the presence of copper, particularly in the 2 to 3 ppm region, where a peak at 2.6 ppm is also shifted to different fields in the paramagnetic protein.

Aromatic region spectra of apoazurin and fully reconstituted azurin were simulated (fig. 4) on the basis of shift and linewidth data for random coil proteins given by McDonald and Phillips [6] and the amino acid composition of azurin [4], assuming that Cu(II) removes the resonances of the tryptophanyl side chain from the aromatic region. The computing procedure of McDonald and Phillips [6] was used.

4. Discussion

It is well known that a paramagnetic ion produces a number of effects in the proton resonance spectrum of residues which it interacts with. The most significant effect brought about by coordination to a paramagnetic center on the proton magnetic resonance of a molecule is a shift of some resonances due to isotropic hyperfine contact and pseudo-contact interaction between the unpaired electron and the nucleus. Moreover, the transverse relaxation time decreases [7] resulting in a broader line-width. This broadening effect might usually be enhanced if the coordinating paramagnetic ion has a quadrupole moment, as in the case of copper.

It should in principle be possible to take advantage of these effects, namely shift and broadening of proton resonances, even in the case of rather complicated systems as metalloproteins, provided that the affected resonances are relatively restricted in number and easily identifiable on the basis of assignment work done on random coil proteins. In the case of azurin a preliminary analysis of its proton magnetic resonance spectrum showed a number of features which make an attempt in this sense reasonable particularly in view of the low α-helix content of this protein [8].

The resonance at 7.7 ppm of the copper-free protein appears as a broad shoulder on the aromatic peak. This broad shoulder may result from the resonance of the C_4 and C_7 protons of the tryptophan indole moiety. In fact, at least in randomcoil protein, these protons have resonances from 7.55 to 7.7 ppm [6]. The computation of the aromatic regions of copper-free and reconstituted azurins (fig. 4) gives support to this assignment, even if the computing procedure of McDonald and Phillips [6], which represents the resonances as triangles, without taking into consideration the resonance wings in the computation of the envelopes, gives rather schematic spectra. As an example, in both computed spectra a' and b' a narrow shoulder due to the resonance of some histidine aromatic protons appears in the high field side (around 7 ppm). This shoulder would disappear if phenylalanine and histidine resonances were removed about 0.05 ppm closer to each other. Such a value is clear below the deviation with which both resonance shifts should be taken, therefore the presence of such shoulders might not be evidenced in a computed spectrum with better averaged shift values.