

The majority of resonances are found between about 10 and -10 ppm but, as can be seen in Fig. 1, several resonances exhibit large hyperfine shifts. Such large shifts indicate that both Co(II) and Ni(II) are in their high-spin state. As expected, the shifts are temperature dependent and Curie-plots for both derivatives show that several of the resonances in Fig. 1 deviate from linearity, indicating some conformational changes at about 30°C that occur in, or near, the metal site. In order to understand this temperature dependent transition, further NMR experiments are needed and the results of such experiments together with the results of magnetic susceptibility measurements will be presented on the poster.

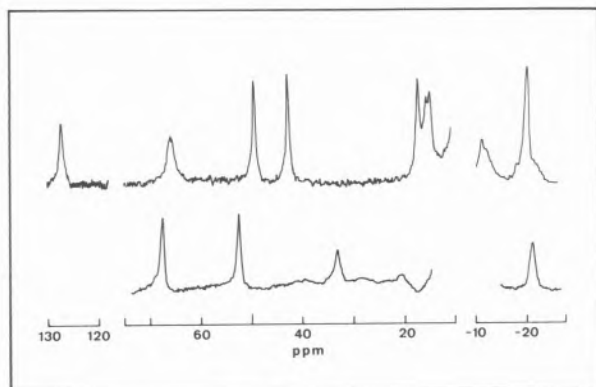


Fig. 1

Hyperfine resonances in the NMR spectra of Co(II)-stellacyanin (upper spectrum) and Ni(II)-stellacyanin (lower spectrum). The part appearing at negative ppm in the upper spectrum has been attenuated twice. Interjacent parts are omitted for clarity. For both derivatives, the concentration was 2 mM in D₂O, pH 7.4. Spectra were recorded using a Bruker WH 270 MHz spectrometer. A «water elimination Fourier transform» (WEFT) pulse sequence was employed

Varying the pH between about 4 and 9 indicated that none of the resonances in Fig. 1 are pH dependent.

It is useful to compare these spectra with the NMR spectra of Ni(II)- and Co(II)-substituted azurin obtained earlier [3]. The differences spotted in the out-shifted regions, in spectra of the two proteins reconstituted with the same metal, might give some clues to the structure of the site in stellacyanin since the fourth ligand in azurin is believed to be a methionine. The NMR spectrum of the Ni(II) derivative does not reveal any hyperfine shifted methyl resonances, thus confirming the absence of methionine in the coordination

sphere of the metal in stellacyanin. However, no clear candidates for the fourth ligand are obvious from the spectra.

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PS3.9 — TH

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CONFORMATION AND TEMPERATURE DEPENDENT SPECTRAL CHANGES IN NICKEL HEMOGLOBIN

INTRODUCTION

It has been shown [1] that normal human adult hemoglobin (HbA) reconstituted with Ni(II) protoporphyrin IX does not bind oxygen and is in a T-like conformation. It was recognized that the value of studies on this stable non-reactive T-like hemoglobin would be greatly enhanced by the pre-

paration of an R-like hemoglobin with the same Ni(II) metal center.

In this paper we demonstrate the formation of such an R-like structure by utilizing the reaction of carboxypeptidase A (CPA). The visible spectra as well as the temperature dependent spectral changes of both of these Ni(II) hemoglobins are compared.

MATERIALS AND METHODS

CPA reacted NiHbA was prepared by the initial treatment of Fe(II)HbA according to the procedure of ANTONINI *et al.* [2] and the subsequent replacement of Fe-porphyrin by Ni-porphyrin [1]. Absolute and difference spectra were recorded on a Cary 14 spectrophotometer. The sample and reference cell holders were thermostated independently using Lauda R-4/RD circulating baths (Brinkman Instruments) and the temperature was measured with a thermister (Yellow Springs Instrument Co., Model 425c).

RESULTS AND DISCUSSION

The T-like NiHbA has two maxima in the Soret region at 398 nm and 420 nm (Fig. 1A). Reaction of CPA with Fe(II)HbA produces only very small perturbations in the visible spectrum in both liganded and unliganded states [3]. However, the CPA treatment produces a major alteration of the Soret band of NiHbA with the peak at 399 nm almost completely eliminated and only one major peak at 420 nm (Fig. 1B). CPA treatment also changes the other visible bands with the 557 nm band replaced by major peaks at 542 nm and 576 nm. The changes in the visible spectrum associated with this reaction suggest an alteration in the metal coordination [4] which does not take place for the Fe(II) hemoglobins undergoing an analogous change in protein conformation [3].

It has previously been shown [5,6] that temperature dependent spectral changes in hemoproteins depend on the internal molecular dynamics and anharmonicity of the heme group and its surrounding atoms.

For Fe(II) hemoglobins this temperature depen-

dence was shown to be 17% smaller in R state hemoglobins than T-state hemoglobins. A comparison of this temperature dependence for R-like and T-like Ni(II) hemoglobins is shown in Fig. 1. Analogous to the results found for Fe(II) hemoglobins the temperature dependence for CPA treated NiHbA is 86% relative to that of the untreated HbA.

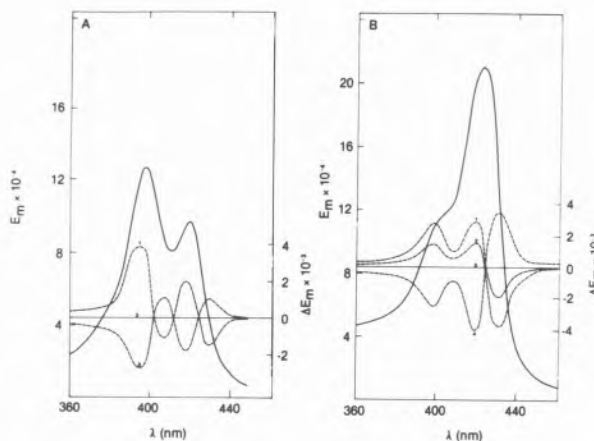


Fig. 1

A) Absorption spectrum (Soret Band) of NiHbA at 21°C-solid line. Temperature induced difference spectra 1,2,3 at 0,21,40°C, respectively.

B) Absorption spectrum (Soret band) of CPA treated NiHbA at 21°C-solid line. Temperature induced difference spectra 1,2,3,4 at 0,10,21,40°C, respectively.

For both A) and B) experimental conditions were concentration per heme 5.8 μ M, reference sample at 21°C, 0.1 M phosphate and pH = 7.00

This finding is a further [4] confirmation that the CPA treated hemoglobin is in an R-like state. Furthermore, the similarity of the magnitude of the temperature dependence for Fe(II) and Ni(II) hemoglobins [6] as well as the similar increase in the temperature dependence associated with the R \rightarrow T transition suggests that this phenomenon is determined by the conformation of the globin in the region of the heme, and not the metal ion coordination which is seen to be different in Ni and Fe hemoglobins.

The studies on stable Ni-hemoglobins which do not bind oxygen in both the R and T conformation are found to be valuable in understanding the relationship between protein conformation and heme environment.

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PS3.10 — MO

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STUDIES ON THE LANTHANIDE COMPLEXES OF SUBTILISINS

A large number of proteins contain strongly bound calcium ions, Ca(II), which are essential for their conformational stability and biological function. The electronic transitions of Ca(II) can not be studied by conventional spectroscopic techniques and this makes it difficult to study the respective binding sites. The trivalent lanthanide ions, Ln(III), possess physico-chemical and spectroscopic properties which make them suitable replacement probes for Ca(II) in calcium-binding proteins.

Subtilisins are a group of extracellular alkaline proteases of bacterial origin. They are stabilized by calcium against autolysis and thermal denaturation. In the present paper circular dichroism (CD) and proteolytic activity determinations were used for studying changes in the conformational and thermal stabilities of four subtilisins; mesentericopeptidase and subtilisins Novo, Carlsberg and DY.

EXPERIMENTAL

Mesentericopeptidase and subtilisin DY were isolated in homogeneous state as described in [1] and [2], respectively. Subtilisins Novo and Carlsberg were received as a gift from Professor IB SVENDSEN (Carlsberg Laboratory, Denmark). The proteolytic activity was determined with casein as substrate. Circular dichroism was measured with a Rousel Jonan Dichrographe III instrument. The thermostability of the enzymes was measured in the sense of heat inactivation at 50°C. The solutions were incubated at 50°C for 24 h in the presence of 10⁻² M CaCl₂ or TbCl₃ or NdCl₃.

RESULTS AND DISCUSSION

The replacement of Ca(II) by Tb(III) or Nd(III) did not affect the dichroic properties and catalytic activity of subtilisins at neutral pH and room temperature, as judged from the CD spectra and proteolytic activity determinations. The retention of the biological activity can serve as an additional evidence that the native conformation has not been appreciably altered due to the substitutions. Fig. 1 shows that the Tb(III) or Nd(III) substitutions have no significant effect on the conformational stability of mesentericopeptidase at acidic pH. The behaviour of the other three subtilisins, after the respective replacements, was similar. The heat inactivation kinetics at 50°C showed that the substitution of the two lanthanides for Ca(II) lowers significantly the stability of all four subtilisins. This is illustrated in the case of mesentericopeptidase in Fig. 2. For example, after 5 h of incubation in the presence of Ca(II) at neutral pH, this enzyme preserves 78% of its caseinolytic activity, but it retains only 15-18% of the initial acti-