RESOLUTION OF SPECIFIC HISTIDINE RESONANCES IN THE 360 MHz ^{1}H NMR SPECTRUM OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE, A 145 000 MOLECULAR WEIGHT PROTEIN, BY PHOTO-CIDNP

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1. Introduction

^{1}H NMR studies of proteins > 50 000 mol. wt are thwarted by the lack of resolution of individual proton signals, even at the highest magnetic fields currently available. This is due both to the enormous number of anisochronous protons present and to the strong line broadening which results from the slow tumbling of macromolecules in solution.

Greatly simplified spectra result from the recently developed photo-CIDNP technique in which a reversible photoreaction of a dye with solvent-exposed tryptophan, tyrosine or histidine residues generates CIDNP effects for specific protons in these residues [1–3]. The technique has already been applied to resolve specific resonances in the NMR spectrum of some relatively small proteins. We now report its application to a large protein. Glyceraldehyde-3-phosphate dehydrogenase (145 000 mol. wt), with its known three-dimensional structure [4,5], was chosen for this purpose. Both the rabbit-muscle and the lobster-tail enzyme were studied.

2. Experimental

Rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase was isolated and purified as in [6]. The lobster-tail enzyme was isolated and recrystallised according to [7]. The spectra shown here were obtained with the NAD^+-containing enzymes; solutions in ^2H_2O contained ~50 mg protein/ml and 25 mM phosphate.

Spectra were recorded on a Bruker HX-360 spectrometer operating in the pulse Fourier transform mode. The sample was irradiated with light pulses from a Spectra Physics model 171 argon ion laser (multiline, 7-W). The difference method for the detection of photo-CIDNP effects in biological macromolecules has been described [1]. As a dye 3-*N*-carboxymethyl-lumiflavin (a gift from Dr F. Müller, Wageningen) was used. Chemical shifts were measured relative to the internal standard sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) or dioxan. The notation pH^x denotes uncorrected pH-meter reading in ^2H_2O solutions.

3. Results and discussion

In fig.1A the 360 MHz proton NMR spectrum of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is shown. The broad envelope seen illustrates the difficulties mentioned above. A photo-CIDNP spectrum was obtained in the presence of 3-*N*-carboxymethyl-lumiflavin, after a pulse from the argon ion laser. The difference spectrum (light minus dark) is shown in fig.1B. Comparison of this difference spectrum with the spectrum of *N*-acetylhistidine under the same conditions makes it likely that at least two histidine side chains contribute to the spectrum. To verify this the pH was varied by adding small amounts of concentrated DCl to the protein solution. The titration curves constructed in this way are shown in

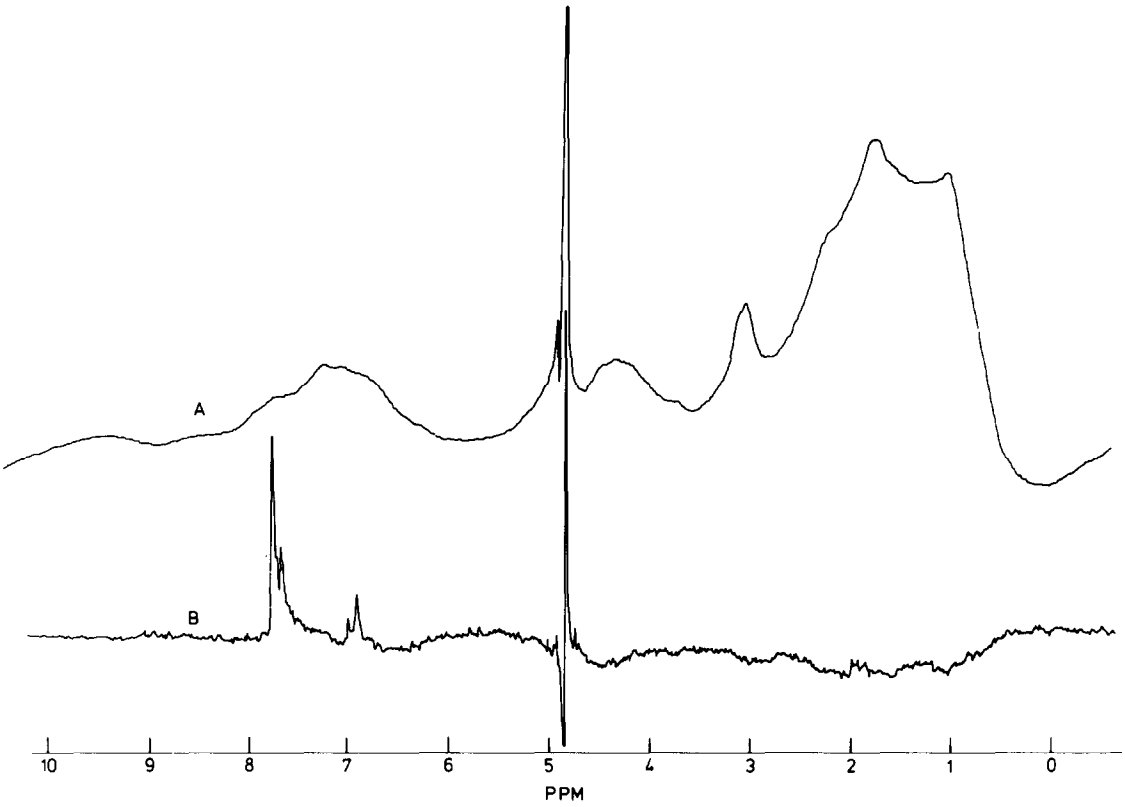

Fig.1. (A) 360 MHz ^{1}H NMR spectrum of 0.4 mM glyceraldehyde-3-phosphate dehydrogenase in ^2H_2O (pH 8.56) 22°C, 25 scans. (B) Photo-CIDNP difference spectrum of the same sample obtained by taking alternating light and dark free induction decays (25 scans). For the light spectrum the sample was irradiated by 0.6 s light pulses from an argon ion laser (7 W, multiline mode).

fig.2. Since all resonances show pH-dependent chemical shifts between pH 5 and 8.5 we assign them to histidine 2,4-ring protons on the surface of the protein. The upper and lower curves in this figure were simulated with pK_a 6.90 and Hill coefficient < 1 ($n = 0.65$) [8]. This suggests that other events titrating in the same pH region also influence the chemical shifts of the ring-proton resonances of the His residue under observation. The middle two curves exhibit no cooperativity ($n = 1$) and pK_a 6.64 was used for their simulation.

Inspection of the X-ray data available [9] revealed that of the 11 histidine residues per monomer of the tetrametric rabbit-muscle enzyme 8 are located on the surface and may thus be accessible to the flavin dye. In the lobster enzyme, however, only 5 histidines are present per monomer, of which His 162 and His 327 are located on the surface. These residues are also present in the rabbit enzyme. The 360 MHz ^{1}H NMR spectrum of the lobster enzyme under the same conditions is very similar to that of the rabbit-muscle enzyme, again being composed of at least two histidines, as could be shown by variation of the pH. We propose, therefore, that His 162 and His 327 are visible in the CIDNP difference spectra both of the rabbit-muscle and the lobster-tail muscle enzymes, but this needs to be proven more directly.

These experiments demonstrate the resolving power of the photo-CIDNP method, even in ^{1}H NMR studies on large macromolecules. The broad envelope shown in fig.1A is a superposition of many broad resonances, as expected for a 145 000 mol. wt protein, but, as shown in the present study, it does also contain sharp components which must be due to enhanced local

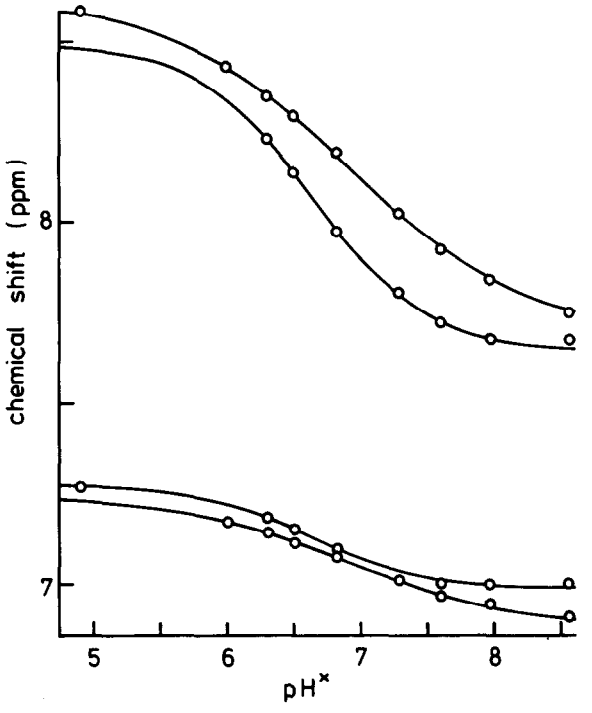

Fig.2. ^{1}H NMR titration curves for the C-2 and C-4 ring protons of two histidine residues in glyceraldehyde-3-phosphate dehydrogenase. Spectra were recorded under the conditions mentioned in the text and in the legends of fig.1B. The continuous lines were calculated according to [8] with pK_a 6.90 (Hill coefficient n = 0.65) for the upper and lower curves and with pK_a 6.64 (n = 1) for the middle two curves.

mobility of some side chains. The tentative assignment of the resolved resonances to the exterior histidines 162 and 327 is based on the data on the location of all amino acid residues in the enzymes from lobster, pig and yeast in [9]. Of these three enzymes only the three-dimensional structure of the lobster holoenzyme is known, so the data on the other enzymes must be treated with some caution. The rabbit and pig enzymes are probably homologous [10].

A similar NMR study on the apoenzymes from the two muscle sources used in this paper was not successful, due to their instability. The apoenzymes from yeast and thermophilic organisms may be easier to study in this respect. Among the residues that probably alter their accessibility for the flavin dye when the coenzyme NAD^+ is removed (cf. [11]), two may be suitable for further CIDNP studies; these two residues are Tyr 317, conserved in different glyceraldehyde-3-phosphate dehydrogenases and in an excellent position to form a charge-transfer complex with the nicotinamide ring of NAD^+ (cf. [4,12]) and His 176, probably directly involved in the actual catalysis [13].

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