^{1}H NMR STUDIES OF TYR-237 OF LACTATE DEHYDROGENASE

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1. Introduction

The usefulness of nuclear magnetic resonance (NMR) spectroscopy for studies of the structure and interactions of proteins is now well-established for small proteins ($M_r \lesssim 30\,000$) where resonances of individual amino acid residues can be resolved and assigned [1–3]. In larger proteins, resolution of individual resonances becomes more difficult, both because of their increased number and because they are broader. ^{13}C-Enrichment [4] and selective deuteration [5] have been used to obtain substantial simplification of NMR spectra of bacterial proteins, while surface aromatic residues can be selectively observed by the photo-CIDNP method [12], but the considerable problem of assignment remains.

We show here that the combination of specific chemical modification with NMR difference spectroscopy makes it possible simultaneously to resolve and assign the ^{1}H resonances of a single tyrosine in lactate dehydrogenase (M_r 140 000).

2. Materials and methods

Pig heart H_4 lactate dehydrogenase was purified by a modification (D. M. P., J. J. H., unpublished) to a method based upon affinity chromatography on oxamate–Sepharose. The enzyme was nitrated by a modification of the method in [6]. Any over-reacted, denatured, protein was removed by repeating the affinity chromatography purification step. The degree of nitration was estimated from the absorbance at 280 and 428 nm, using the extinction coefficients in [6]. The A_{280} value was corrected as in [7]. The preparation contained 0.9–1.0 mol nitrotyrosine/mol enzyme subunits and ≥85% of the nitrogroups were on residue 237 [8]. The specific enzyme activity of the nitrated enzyme was 275 μmol NADH oxidised . min^{-1} . mg protein^{-1} at 1 mM pyruvate. This compares to 360 μmol NADH oxidised . min^{-1} . mg^{-1} for the native enzyme at 0.3 mM pyruvate (both with 0.12 mM NADH at pH 7.2 and 25°C).

Samples (0.2 g) of either native or nitrated enzyme were sedimented from suspension in $(NH_4)_2SO_4$, and dissolved in a solution (2 ml) prepared from Na_2HPO_4 (99.4 mg), KH_2PO_4 (40.8 mg) and ^2H_2O (99.8 atom % ^{2}H), pH (meter reading) 7.5. Each sample was dialysed against seven 5 ml portions of the same buffer over 2 days at 0°C. The final enzyme subunit levels were 1.64 mM (native enzyme) and 1.44 mM (nitrated enzyme). The binary complex was prepared by adding 0.9 mol NADH/mol subunits. The ternary complex was prepared by adding Na-oxamate to the binary complex solution to give a final concentration of 2.7 mM.

270 MHz ^{1}H NMR spectra were obtained by the Fourier transform method using a Bruker WH270 spectrometer. For each spectrum 3000 transients were averaged using quadrature detection. The pulse interval was 0.5 s, acquiring 4096 data points for a spectral width of 4.2 kHz. Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to line broadening of 4 Hz, and 4096 zeros were added to give a total data table of 8192 points (digital resolution of 1 Hz/point). The sample temperature was 22 (±1)°C.

3. Results and discussion

The aromatic region of the 270 MHz ^{1}H NMR spectrum of lactate dehydrogenase is shown in fig.1A.

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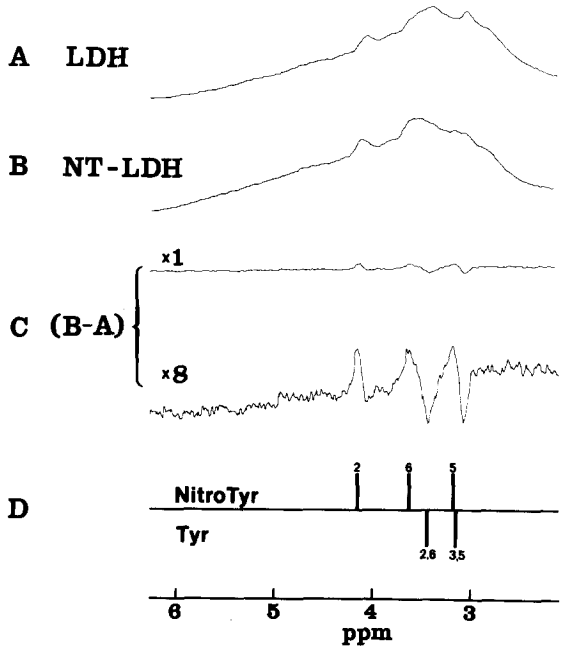

Fig.1. The aromatic region of the 270 MHz ^{1}H NMR spectrum of native and nitrotyrosyl$_{237}$ pig heart lactate dehydrogenase: (A) native enzyme; (B) nitrotyrosyl$_{237}$ enzyme; (C) difference spectrum B–A, shown at the same and at an 8-fold expanded vertical scale; (D) schematic representation of the spectra of tyrosine and nitrotyrosine.

The revised sequence of lactate dehydrogenase of pig heart contains 7 tyrosine, 5 phenylalanine, 6 tryptophan and 7 histidine residues/subunit [9]. Thus each of the 4 equivalent subunits will contribute resonances from 97 protons (together with those of any slowly exchanging NH protons) to this region of the NMR spectrum. As expected from a protein of this size, only a broad envelope of overlapping resonances is observed. (Neither in the aromatic nor in the aliphatic regions of the spectrum are there any very sharp signals which would be expected if there were regions of the protein backbone which were flexible on the timescale of 10^{-8} s.)

The spectrum is not entirely featureless, however, and on comparison with the spectrum of nitrotyrosyl$_{237}$ lactate dehydrogenase (NT-LDH; fig.1B) a number of differences can be discerned. The difference between the two spectra is shown in fig.1C. Three positive and 2 negative peaks are evident, and these can be assigned to the 3 one-proton resonances of nitrotyrosine and the 2 two-proton resonances of tyrosine, respectively, by comparison with the chemical shifts of the free amino acids, shown in fig.1D. The intensities of these signals in the difference spectrum are approximately those expected, from fig.1A,B, for a single tyrosine or nitrotyrosine residue/subunit (although the partial overlap of some of the positive and negative signals will decrease their apparent area). We conclude, therefore, that the negative signals in the difference spectrum at 3.42 and 3.07 ppm can be assigned to the 2,6- and 3,5-protons, respectively, of Tyr 237. Similarly, the positive signals at 4.16, 3.64 and 3.18 ppm arise from the 2-, 6- and 5-protons of nitrotyrosine 237 in the modified enzyme. This is the first time a clear resonance assignment has been possible in the NMR spectrum of a protein as large as lactate dehydrogenase.

In the crystal [9], the aromatic ring of Tyr 237 lies on the surface of the protein, and it is clear from fig.1 that this is also the case in solution. The difference in chemical shift between the resonances of the protein and the corresponding ones of the free amino acid are very small for all 5 resonances in the difference spectrum ($\leqslant$0.02 ppm for all except the 3,5-proton resonance, for which the difference is 0.08 ppm). This is obviously consistent with a solvent-like environment for these protons in the protein. The equivalence of the 2- and 6-protons and of the 3- and 5-protons of Tyr 237 would be expected if the aromatic ring was surrounded by solvent, but is also seen in many cases for 'buried' residues, due to rapid 'flipping' about the C_β–C_γ bond [10,11]. The tyrosine resonances are relatively sharp (25–40 Hz), which may indicate significant freedom of motion of the ring on the surface of the protein – and which will also help to account for the fact that these resonances are fairly easy to observe, not only in the difference spectrum but also in the spectra of fig.1A,B.

The simplicity of the difference spectrum (fig.1C) clearly shows that no other aromatic residue of the protein is perturbed by nitration of Tyr 237, and from the aliphatic region of the difference spectrum (not shown) it appears that $\lesssim$10 protons/subunit in other residues are perturbed. The use of this nitrotyrosyl group as a 'reporter group' in optical spectroscopy will therefore not be invalidated by major perturbations of the native structure of the protein.

Tyr 237 is close to the 'loop' (residues 98–120) whose conformation in the crystal changes significantly on forming the ternary complex [9]. Changes in the optical absorption of the nitrotyrosyl$_{237}$ chromophore which occur on the binding of NADH and

oxamate (D. M. P., J. J. H., unpublished) have been attributed to this conformational change. The differences in the NMR spectra between the apoenzyme and the binary or ternary complexes are too extensive and complex to be readily interpreted. However, it is possible, by using the difference between nitrotyrosyl and native enzyme spectra, to examine the effects of ligand binding on Tyr 237 specifically.

The difference between the NMR spectra of the NADH complexes of the nitrotyrosyl and native enzyme was indistinguishable from that shown in fig.1C for the apoenzyme, showing that coenzyme binding has no effect on this residue (in agreement with the results of optical studies; [D. M. P., J. J. H., unpublished]). In the difference between the spectra of the two ternary NADH + oxamate complexes, the negative peaks from the tyrosine residue could clearly be seen, but the positive peaks from the nitrotyrosyl residue could not, suggesting that they must be broader in the ternary complex than in the apoenzyme or binary complex. There are two obvious possible origins of this broadening:

(i) If, as suggested above, this nitrotyrosine residue has appreciable motional freedom in the apoenzyme, then its resonances would broaden if this motional freedom were substantially decreased in the ternary complex. Since the resonances of the tyrosine residue in the native enzyme do not broaden, this would imply the existence of some interaction specific for the modified nitrotyrosine.

(ii) The possibility of exchange broadening is raised by the observation (D. M. P., J. J. H., unpublished) of a slight but significant change in the pK of nitrotyrosine$_{237}$ on forming the ternary complex, such as to bring the pK closer to the pD of the sample (although the deuterium isotope effect on the pK is not known). If the rate of exchange between the protonated and unprotonated forms of the nitrotyrosine residue were not very fast, then this could lead to broadening of its resonances. Attempts to confirm that this was the origin of the observed broadening were frustrated by precipitation of the protein at pH $\leqslant$6.

Whichever explanation for the effects on the nitrotyrosyl resonances is the true one, the difference spectra do show that the effects of ternary complex formation on Tyr 237 in the native enzyme are minimal, since its resonances shift by $<$0.03 ppm. The fact that a larger effect is observed on the modified than on the native residue may reflect different interactions of the nitrotyrosyl group with neighbouring residues or simply the fact that its lower pK (7.0) makes its proton chemical shifts (and its optical absorption spectrum) more sensitive to slight changes in its electrostatic environment.

In either event, these experiments illustrate the fact that the use of NMR difference spectroscopy in conjunction with specific chemical modification has the powerful advantage for studies of the solution conformation of enzymes that both the modified residue and its native counterpart can be observed selectively, even in a protein of M_r 140 000, at least when the residue in question has some mobility relative to the protein as a whole.

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