Carbon-2 Proton Exchange at Histidine-41 in Bovine Erythrocyte Superoxide Dismutase

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The C-2 proton of one histidine residue in bovine erythrocyte superoxide dismutase is shown to be particularly labile. This residue is identified by tritiation, protein digestion and subsequent peptide 'mapping' as histidine-41. A half-life for the exchange of histidine C-2 ^1H for ^2H in $^2\text{H}_2\text{O}$ as solvent, at pD8.1 and 40°C , is estimated as approx. 9.2 h, by ^1H nuclear-magnetic-resonance spectroscopy.

The exchange of C-2 protons of histidine residues in a variety of proteins has previously been noted (Markley & Cheung, 1973; Ohe *et al.*, 1974; Krieger *et al.*, 1976). The process has usually been investigated by the use of ^3H labelling or n.m.r.† spectroscopy. In the present work we have used both these methods to characterize the relatively rapid exchange of a histidine C-2 proton in bovine erythrocyte superoxide dismutase (EC 1.15.1.1) and to identify the specific residue involved.

Materials and Methods

Superoxide dismutase (90 mg), prepared by the method of Bannister *et al.* (1971), was incubated in a solution containing 20 mM-sodium phosphate buffer, pH 8.6, 1 M-NaCl and 100 mCi of $^3\text{H}_2\text{O}$ (The Radiochemical Centre, Amersham, Bucks., U.K.), in a total volume of 2 ml, at 40°C for 4 h. The protein was then reduced, carboxymethylated and digested with pepsin by the method of Steinman *et al.* (1974). The digest was freeze-dried and the peptic peptides were separated from undigested dismutase and pepsin on a column (1.5 cm $\times$ 80 cm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated with 1% (v/v) formic acid. The resulting mixture of peptides was then 'mapped' by two-dimensional paper chromatography/high-voltage electrophoresis at pH 6.5. Histidine-containing peptides were detected by spraying with Pauly's reagent (diazotized sulphanilic acid). Regions of the 'map' that showed a positive reaction for histidine were cut out and immersed in a mixture containing 150 ml of toluene, 50 ml of 2-methoxyethanol and 1 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole,

and their radioactivity was measured by liquid-scintillation counting. The peptides from corresponding radioactive spots on a second identical map, stained with 0.2% ninhydrin, were eluted with 2% (v/v) NH_3 solution and freeze-dried.

The eluted peptides were hydrolysed with 6 M-HCl at 105°C for 24 h. Analysis of the hydrolysate was performed on a JEOL model 6HA amino acid analyser.

The experimental conditions for recording n.m.r. spectra were as described previously (Cass *et al.*, 1977). The sample for which kinetic data are reported consisted of superoxide dismutase (5 mM), reduced with the minimum amount of solid sodium dithionite (about 1 mg), sodium phosphate buffer, (20 mM) and NaCl (1 M) dissolved in 99.7% $^2\text{H}_2\text{O}$. The pH values of $^2\text{H}_2\text{O}$ solutions are quoted as direct meter readings (pD).

Results and Discussion

A portion of the 270 MHz ^1H n.m.r. spectrum of the dithionite-reduced enzyme is shown in Fig. 1(a). We have previously (Cass *et al.*, 1977) given a more detailed discussion of assignments in the n.m.r. spectrum than will be given here. In the region of the spectrum shown in Fig. 1(a) four of the eight histidine C-2 ^1H resonances occur and are labelled 1–4. The remaining intensity in the region shown is due to the protons of NH groups, most of which are relatively inert to exchange with solvent. Over a period of hours at pD8.1 and 40°C , a selective decrease in the intensity of peak 1 is observed (Figs. 1b and 1c) corresponding to ^1H to ^2H exchange on C-2 of a single histidine residue. The additional intensity apparent in Fig. 1(c) is derived from the exchange of some NH protons. A pseudo-first-order plot, fitted by the method of least squares,

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† Abbreviation: n.m.r., nuclear magnetic resonance.

Fig. 1. ^1H n.m.r. spectra (270MHz) of the low-field region of the dithionite-reduced Cu(I)/Zn(II) superoxide dismutase. Experimental details are given in the text. (a) pD 8.1; (b) pD 8.1 after 7.2h; (c) difference (a) - (b).

Table 1. Amino acid composition of tritiated peptides H1 and H2

	Amino acid composition (mol/100mol)	
	H1	H2
Lys	0	2.03
His	0.80	1.53
Arg	0	*
Asp	0.89	3.73
Thr	0.97	1.32
Glu	1.35	3.13
Gly	2.29	2.17
Ala	0	1.37
Val	0	3.14
Leu	0.79	1.00
Phe	0.91	*

* Detected in small amounts, <0.4.

yields a rate constant of 0.075 h^{-1} and a corresponding half-life of 9.2h. The exchange is slower at pD 7.5 and more rapid at pD 8.9.

Radiolabelling, reduction and carboxymethyl-

ation, followed by digestion with pepsin and separation of the peptides, yielded a peptide 'map' in which only two spots (denoted H1 and H2) showed radioactivity appreciably above background. Uncorrected counts after elution with NH_3 were 274 and 100 c.p.m. respectively as compared with a background of 7 c.p.m. (average of three 20 min counts). From the amino acid composition given in Table 1, it can be deduced that spot H1 contains a single octapeptide corresponding to the fragment P3 reported by Steinman *et al.* (1974). This peptide, Leu-Thr-Glu-Gly-Asp-His-Gly-Phe, is derived from positions 36-43 in the amino acid sequence of the protein. Spot H2, although not fully characterized, being proximate to spot H1 in the peptide 'map', probably consists of a mixture of oligopeptides, one component corresponding to peptide P4 (residues 37-43) described by Steinman *et al.* (1974).

It is concluded that the labile C-2 proton is derived from histidine-41. Peak 1 in Fig. 1 is therefore assigned to this proton. This is consistent with the n.m.r. assignment proposed earlier from less direct evidence (Cass *et al.*, 1977).

A number of investigations of the exchange rates of histidine C-2 protons have been reported (Markley & Cheung, 1973; Ohe *et al.*, 1974; Krieger *et al.*, 1976) for a variety of proteins and a variety of experimental conditions. Half-lives observed varied from approx. 30h to more than 50 days. Although none of these investigations were conducted under conditions identical with those of the present investigation, it is apparent that the exchange for histidine-41 in bovine erythrocyte superoxide dismutase is comparatively rapid. For example, half-lives for histidines-12, -48, -105 and -119 in ribonuclease at pH8.0 and 40°C (Markley, 1975) are 4.55, 43, 1.43 and 2.02 days respectively.

Histidine-41 is in a region of β -pleated structure and appears to be relatively inaccessible to solvent (Richardson *et al.*, 1975). However, it is plausible that residues in the vicinity of the imidazole ring of histidine-41 may serve as intramolecular catalysts in the slow deprotonation step, or the local environment may stabilize the intermediate ylide postulated in the exchange mechanism (see e.g. Markley & Cheung, 1973).

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