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The formation of dehydroalanine residues in alkali-treated insulin and oxidized glutathione

A nuclear-magnetic-resonance study

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^{1}H- and ^{13}C-n.m.r. measurements enable direct observation of the rate of formation of dehydroalanine residues resulting from lysis of the disulphide bonds of insulin and oxidized glutathione in base at pD 13. The data provide clear evidence for the β-elimination mechanism for this reaction. The dehydroalanine-containing products from the lysis of insulin undergo secondary reactions.

Two mechanisms are commonly invoked to explain lysis of the disulphide bonds of proteins or low-molecular-weight model compounds in alkali:

$$2\,R\text{–}S\text{–}S\text{–}R + 4\,OH^- \rightleftharpoons 2\,R\text{–}S^- + 2\,R\text{–}SOH + 2\,OH^- \rightleftharpoons 3\,R\text{–}S^- + R\text{–}SO_2^- + 2\,H_2O \quad (1)$$

$$R\text{–}S\text{–}S\text{–}CH_2\text{–}\underset{\displaystyle R}{\overset{\displaystyle R}{\underset{|}{\overset{|}{C}}}}\text{–}H + OH^- \rightleftharpoons R\text{–}S\text{–}S^- + CH_2{=}C\begin{matrix}R\\R\end{matrix} + H_2O \quad (2)$$

Several studies have been reported that support one or other of these mechanisms [Schneider & Westley (1969); Donovan & White (1971); for reviews see Cecil & McPhee (1959) and Danehy (1966)]. We have recently reported that the lysis of the disulphide bonds of insulin in alkali or by thiol compounds proceeds by the β-elimination mechanism (2) (Helmerhorst & Stokes, 1983).

Lanthionine (Horn *et al.*, 1941), lysinoalanine (Bohak, 1964), ornithinoalanine (Ziegler *et al.*, 1967) and β-aminoalanine (see Asquith & Carthew, 1972*a*) have been identified as products of alkaline degradation in various proteins. It is postulated that amines (e.g. ε-amino groups of lysine residues) or thiol add across the double bond of dehydroalanine to form these products. In addition, the formation of pyruvate, which is presumed to arise from the decomposition of dehydroalanine during acid hydrolysis, has been reported (Gawron & Odstrchel, 1967). γ-Glutamyldehydroalanylglycine has been synthesized from GSH. The isolated pure product has been identified by ^{1}H n.m.r. as the same as that produced when GSSG is treated with alkali (Asquith & Carthew, 1972*b*). However, to our knowledge, no reports have appeared providing direct evidence to substantiate the generation of dehydroalanine residues by alkaline cleavage of disulphide bonds in proteins. N.m.r. spectroscopy affords an ideal method for monitoring the formation of olefinic dehydroalanine residues in proteins. The ^{1}H-n.m.r. spectra of proteins generally exhibit few resonances in the region 4.5–6.5 p.p.m., which characterizes olefinic moieties (Wüthrich, 1976). In the β-elimination mechanisms (2) of the lysis of disulphide bonds the formation of dehydroalanine residues containing the olefinic function $=CH_2$ has been proposed. In the present paper we show that the formation and rate of formation of dehydroalanine residues in alkali-treated insulin and GSSG can be followed *in situ* by using n.m.r. spectroscopy.

Abbreviations used: GSSG and GSH, oxidized and reduced glutathione respectively.

Experimental

Materials

Single-peak pig zinc insulin [0.3% (w/w) zinc; 25.7 units/mg] was obtained from the Commonwealth Serum Laboratories (Melbourne, Vic., Australia). NaO^2H [40% (w/v) in ^2H_2O] was

obtained from Merck, Sharpe and Dohme (Point Claire-Dorval, Que., Canada), and ^{2}HCl [20% (w/v) in ^2H_2O] from Merck (Darmstadt, West Germany). These solutions were diluted appropriately to 0.1 or 0.2M with 99.75% ^2H_2O obtained from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, N.S.W., Australia). GSSG was prepared by the procedures described by Asquith & Carthew (1972*b*). All other materials were of A.R. grade and were obtained commercially.

Preparation of sample for ^{1}H- and ^{13}C-n.m.r. studies

^{1}H- and ^{13}C-n.m.r. spectra were measured at 270 and 67.89MHz respectively in the Fourier mode with a Bruker HFX-270 spectrometer. All solutions for n.m.r. study were prepared by initially dissolving the peptide in an appropriate volume of ^{2}HCl (0.1M), followed rapidly by NaO^{2}H (0.2M) to adjust to the appropriate pH. Total solution volume was 0.5ml with concentrations at 33mM and 2.5mM for GSSG and insulin respectively for ^{1}H-n.m.r. measurements in 5mm tubes, and 1.5ml with concentrations at 90mM and 12mM for GSSG and insulin respectively for ^{13}C-n.m.r. measurements in 10mm tubes. Reaction time courses were determined from the point of addition of the base. ^{1}H-n.m.r. spectra were determined in a sequential manner, with suppression of the water resonance by using a gated decoupler pulse of 0.2s duration. A total of 256 or 1024 transients were averaged per spectrum, with a sweep width of 3600Hz and 4096 data points. The acquisition time for each spectrum in the time course ranged from 3.28 to 13.08min. In general, the acquired free induction decays were Fourier-transformed with line-broadening (1Hz) and zero-filling (to 8192 data points). The areas and amplitudes of time-dependent peaks were normalized within a given data set and were measured by using standard procedures. Chemical shifts (in p.p.m.) are quoted relative to sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (in ^2H_2O) present in a capillary inside the n.m.r. tube. ^{14}C-n.m.r. spectra were also determined sequentially, by averaging 1024 or 16384 transients per spectrum with a sweep width of 15000Hz and 16384 data points, in 92 or 147.5min per spectrum. ^{13}C chemical shifts (in p.p.m.) are quoted relative to tetramethylsilane, though they were measured by addition of dioxan (5μl) to the solutions.

Results and discussion

GSSG

In the present study GSSG was used as a model compound. The initial ^{1}H-n.m.r. spectrum of GSSG at pD13 exhibits five distinct resonances, at 3.75, 3.24, 2.93, 2.37 and 1.86p.p.m. to the Gly $C_{(\alpha)}H_2$, Cys $C_{(\beta)}H_2$, Cys $C_{(\alpha)}H$, Glu $C_{(\gamma)}H_2$ and Glu $C_{(\beta)}H_2$ protons respectively (Kuchel, 1981). After only a few minutes' reaction at pD13, new resonances, which increased in amplitude with time, were detected at 5.71 and 5.54p.p.m., as shown in Fig. 1. These resonances were attributable to the γ-glutamyldehydroalanylglycine methylene protons (Asquith & Carthew, 1972*a*,*b*). [Asquith & Carthew (1972*a*,*b*) incorrectly defined these resonances as a 'doublet'. Rather, the signals arise from single lines characterizing the *E* and *Z* protons of the olefinic methylene group.] An additional singlet appeared with time at 3.80p.p.m., which we attribute to the glutathione persulphide ion, GSS$^-$ (see Helmerhorst & Stokes, 1983). During the course of the reaction considerable changes in the multiplicities about 2.7–3.3p.p.m. were observed. From several time-course experiments (up to 5h reaction time) it was apparent from the amplitude of the characterizing signals at 5.71, 5.54, 3.80 and 3.75p.p.m. that 1mol of GSS$^-$ and 1mol of γ-glutamyldehydroalanylglycine were produced per mol of GSSG. We suggest from this direct evidence that, in contrast with the observations reported by Asquith & Carthew (1972*b*), the reaction at pD13 does not yield predominantly γ-glutamyldehydroalanylglycine. It is noteworthy that on addition of KCN the quantities of the two major products are quenched.

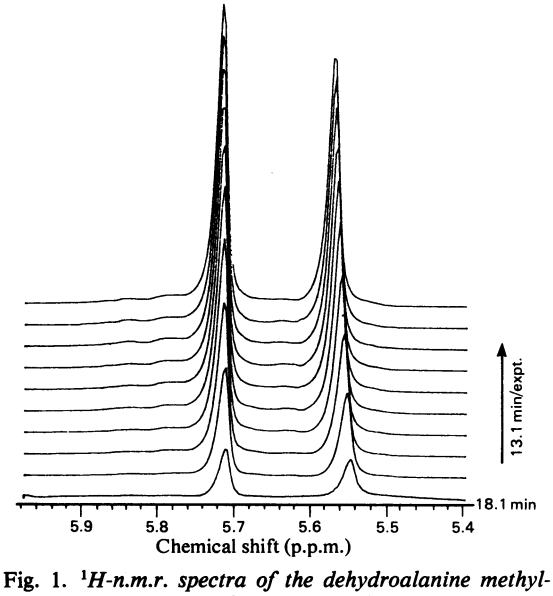

Fig. 1. *^{1}H-n.m.r. spectra of the dehydroalanine methylene proton region in the reaction of GSSG with base, showing the increase in amplitude with time*

The Figure shows the olefinic region of the ^{1}H-n.m.r. spectrum of a 32mM solution of GSSG in NaO^{2}H at pD13. The initial spectrum was determined at 18.1min after mixing; subsequent spectra were collected at 13.1min intervals under computer control.

Table 1. *^{13}C chemical shifts of GSSG and products of reaction with base at pD 13*

Position of C atom	Chemical shift (p.p.m.) GSSG	γ-Glutamyldehydroalanylglycine	Other*
Gly $C_{(\alpha)}$	43.37	43.37	43.37
Cys $C_{(\alpha)}$	53.58	135.42	(53.09, 52.98)
Cys $C_{(\beta)}$	38.79	112.80	(38.79, 37.96)
Glu $C_{(\gamma)}$	32.28	(33.48)	(32.63)
Glu $C_{(\beta)}$	30.74	30.74	30.48
Glu $C_{(\alpha)}$	55.55	55.55	55.55
Cys CO	171.74	(172.83, 172.26)	171.74
Gly CO	176.29	176.29	(175.49)
Glu αCO	182.26	182.26	182.26
Glu γCO	176.04	176.04	176.04

* Resonances attributable to γ-glutamyldehydroalanylglycine derivative and/or GSS^-. Parentheses indicate several low-intensity resonances about this region attributable to reaction products.

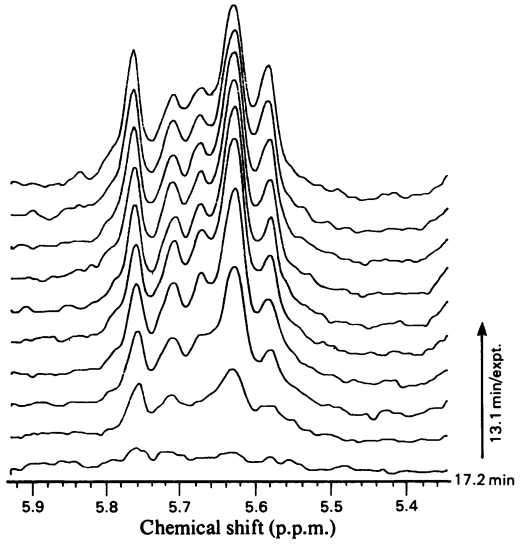

Fig. 2. *^1H-n.m.r. spectra of the dehydroalanine methylene proton region in the reaction of insulin with base at pD 13*

The Figure shows the olefinic region of the ^1H-n.m.r. spectra of a 2.5 mM solution of insulin in NaO^2H at pD 13. The initial spectrum was the result of accumulation of 1024 transients, 17.2 min after initial mixing. Subsequent spectra were collected at 13.1 min intervals under computer control.

Acidification of the reaction solutions results in pH-induced shifts of the characterizing signals to 5.74, 5.69 ($\Delta\delta$ 0.05 p.p.m.) and 4.04 p.p.m. H_2S was simultaneously generated.

The ^{13}C-n.m.r. spectra of GSSG at pD 13 confirmed the assignment of the proton olefinic resonances. Ten distinct lines were observed in the initial spectrum (zero time). The assignments are given in Table 1, and are made by following Feeney *et al.* (1974) and Jung *et al.* (1973) for GSSG and GSH, in particular noting their observations on chemical-shift cross-over with pH. With time the resonances at 38.79 and 53.58 p.p.m., assigned to the Cys $C_{(\beta)}$ and Cys $C_{(\alpha)}$ carbon atoms, are decreased in magnitude, with concomitant observation of lines at 135.42 and 112.2 p.p.m. characterizing the γ-glutamyldehydroalanylglycine $>C=H_2$ moiety during the course of the reaction (Breitmaier & Voelter, 1974). Evidence for the formation of GSS^- is less obvious in the ^{13}C-n.m.r. spectrum than in the ^1H-n.m.r. spectrum but the presence of GSS^- can be deduced from the relative intensities of lines associated with the Gly $C_{(\alpha)}$ and Glu $C_{(\alpha)}$ carbon atoms (Table 1).

Insulin

Time-course ^1H-n.m.r. spectra of insulin in base at pD 13 are shown in Fig. 2. The broad multiplet nature of the olefinic resonances contrasts with that observed in the GSSG spectra (Fig. 1), and demonstrates the non-equivalence of the product dehydroalanine residues. As a control experiment, a time course of the ^1H-n.m.r. spectrum of insulin at pD 11 was determined. The spectra exhibit only minor intensity changes in the aromatic region (6.5–7.9 p.p.m.), but no increase above noise was observed in the olefinic region over a 4 h period.

Two low-field singlets at 7.55 and 7.70 p.p.m. observed in insulin solutions at pD 11 or initially at pD 13 were assigned to the H-2 protons of the histidine residues 5 and 10 of the B-chain. These signals coalesced to a broad multiplet centred at 7.65 p.p.m. when insulin solutions were denatured in 7.5 M-urea or incubated at pD 13. Analogous changes were also observed in the aromatic region of the spectra, these reflecting the slow loss of tertiary structure in the insulin molecule as it denatures in 7.5 M-urea or in base at pD 13 during lysis of the disulphide bonds.

By using the low-field histidine signals to normalize the ^1H-n.m.r. spectra, we observed that up to 2.5 dehydroalanine residues/molecule were produced as the disulphide bonds in insulin were cleaved at pD 13. The lag in appearance of the olefinic resonances on addition of base to insulin solutions was abolished with prior denaturation of the insulin in 7.5 M-urea, as shown in Fig. 3. Olefinic peak amplitude passes through a maximum, then declines as reaction(s) that consume the labile dehydroalanine moieties exceeded their rate of formation. Similar changes were observed by Helmerhorst & Stokes (1983) when the reaction mixture was

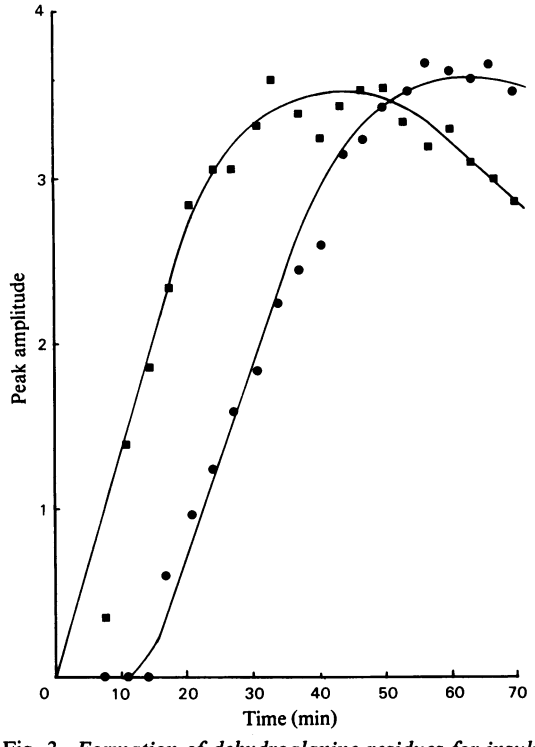

Fig. 3. *Formation of dehydroalanine residues for insulin in base at pD 13*

The change in amplitude is shown, as a function of time, of the dehydroalanine methylene proton resonances, relative to the histidine resonance (internal reference) in insulin in NaO^2H at pD 13 (●), and insulin first denatured at 7.5 M-urea then treated with base at pD 13 (■).

assayed for persulphide residues. These observations preclude quantitative determination of the number of disulphide bonds exposed to lysis in native and denatured insulin (Blundell *et al.*, 1972).

The complexity of the ^{13}C-n.m.r. spectrum of insulin at pD 13 and the relative insensitivity to detection of natural-abundance ^{13}C nuclei precludes observation of a ^{13}C-n.m.r. spectrum at zero time. Rather, the ^{13}C-n.m.r. spectrum of insulin at a pD (11.5) at which the protein is stable (Bradbury & Brown, 1977) must be compared with that obtained in a minimum reaction/acquisition time (approx. 2 h) at pD 13, when denaturation (evidenced by sharpening of lines) and product formation have already occurred. Observation of additional resonance lines in the range 111–117 p.p.m. matches well with the value observed for the dehydroalanine olefinic methylene group in γ-glutamyldehydroalanylglycine (112 p.p.m.). However, relative sensitivity prevents detection of the quaternary carbon atoms corresponding to these functions.

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