Biochemical Pharmacology, Vol. 51, pp. 1259–1267, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02408-5

The Formation and Stability of Imidazolidinone Adducts from Acetaldehyde and Model Peptides

A KINETIC STUDY WITH IMPLICATIONS FOR PROTEIN MODIFICATION IN ALCOHOL ABUSE

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ABSTRACT. The kinetics of the reaction of acetaldehyde (AcH) with the α-amino group of several di- and tripeptides to form 2-methylimidazolidin-4-one adducts were determined at pH 7,4, 37°C, using reverse phase HPLC to separate peptides from adducts. The imidazolidin-4-one structure of the adducts was confirmed by ^{13}C NMR spectroscopy. The reaction of val-gly-gly with AcH was shown to follow second-order kinetics over a wide range of concentrations of both reactants, with $k_2 = 0.734 \pm 0.032\ M^{-1}\ min^{-1}$. Under conditions similar to those in the liver of an alcoholic during chronic ethanol oxidation ($[AcH]_o$ = 50–910 μM; [free peptide α-amino groups]$_o$ = 1.5 mM), the reaction proceeded until effectively all of the AcH had been consumed. The side chain of the N-terminal amino acid was shown not to have a marked effect on the rate of imidazolidinone formation. The decomposition of the imidazolidinone adduct of val-gly-gly and AcH was observed at 60–100°C. Extrapolation of an Arrhenius plot to 37°C provided an estimate of k_{obs} of 0.002 h^{-1} ($t_{1/2}$ ~ 14 days). Based on these kinetic studies, it is concluded that imidazolidinone adducts of AcH with proteins may be present in the liver and, possibly, in the blood of alcoholics. BIOCHEM PHARMACOL 51;10:1259–1267, 1996.

KEY WORDS. acetaldehyde; peptides; amino groups; imidazolidinone adducts; alcohol abuse; modified proteins in alcoholism

AcH,† the primary metabolite of ethanol, reacts *in vitro* with peptides and proteins to give stable and unstable covalent adducts [1–3]. There is considerable current interest in these adducts because of their possible involvement in both acute and chronic effects of alcohol ingestion. San George and Hoberman [4] studied the reaction between AcH and haemoglobin *in vitro* at pH 7.4 and 37°C. Under these conditions, the major stable adducts (defined as being stable to dialysis at pH 7.4 for 2 days at 4°C) were shown to be the 2-methylimidazolidin-4-ones formed by reaction at the N-terminal valine residues of the peptide chains, particularly the β-chains.

In earlier studies on imidazolidinones, du Vigneaud and his coworkers [5] showed that acetone reacts with the N-terminal cysteine residue of the nonapeptide hormone oxytocin to form the relatively stable 2,2-dimethylimidazolidinone derivative; and Summers *et al.* [6, 7] showed that a similar reaction occurs between AcH and the pentapeptides methionine-enkephalin and leucine-enkephalin. In both of these examples, the imidazolidinones (which are themselves inactive as hormones) decompose slowly under *in vivo* conditions to regenerate the active hormones. This raised the possibility that imidazolidinones may be useful as prodrugs, an idea that has been developed by Klixbüll and Bundgaard [8, 9] in their work on imidazolidinone formation from ampicillin and peptides. Imidazolidinone adducts are also formed by reaction of AcH with a metabolite of lidocaine, a reaction that appears to occur *in vivo* in monkeys treated with both lidocaine and ethanol [10].

To allow further comment about the possible formation and accumulation of imidazolidinone adducts between AcH and proteins *in vivo* after ethanol ingestion, we need to know how rapidly and to what extent imidazolidinones form under *in vivo* conditions, and how stable they are. However, very little kinetic and thermodynamic data are available on the reaction of AcH with peptides and proteins. Summers and Lightman [11] determined a $t_{1/2}$ of 14 min for imidazolidinone formation between AcH (73 mM) and methionine-enkephalin (3.6 mM) at pH 7.0, 25°C. They also reported that the adduct decomposed rapidly in 1% acetic acid at 100°C, but only very slowly at pH 7, 25°C. San George and Hoberman [4] found that transfer of the AcH moiety occurred when the imidazolidinone of one

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† *Abbreviations:* AcH, acetaldehyde; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

Received 9 July 1995; accepted 5 December 1995.

peptide was equilibrated with a large excess of a second peptide, a reaction that is potentially important *in vivo*. They did not, however, estimate the rate of this transfer reaction. In the present paper, we describe a kinetic study of the formation and stability of imidazolidinones from AcH and several di- and tripeptides.

MATERIALS AND METHODS

Materials

The following compounds were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.): L-valylglycylglycine (val-gly-gly), L-alanylglycylglycine (ala-gly-gly), glycylglycyl-L-valine (gly-gly-val), L-phenylalanylglycylglycine (phe-gly-gly), glycyl-L-leucine (gly-leu), glycyl-L-leucinamide (gly-leu-NH_2), α-N-acetyl-L-lysine N-methylamide, NADH disodium salt, yeast alcohol dehydrogenase and TNBS. The TNBS was recrystallized from HCl solution as described by Fields [12]. AcH was obtained from BDH Chemicals Co. (Kelsyth, Victoria, Australia), with a minimum assay of 99%. Labelled AcH (97.9 atom % [1,2-^{13}C]AcH) was purchased from MSD Isotopes Canada Inc. (Toronto). D_2O (99.78 atom %) was obtained from Miles-Yeda Laboratories (Elkhart, IN). 3-(Trimethylsilyl)propanesulfonic acid sodium salt was obtained from Wilmad Glass Co. (Castlehill, NSW, Australia).

HPLC Separations

Peptides and imidazolidinone adducts were separated by isocratic reverse phase HPLC in 54 mM phosphate buffer, pH 3.1, on a Waters C18 μBondapak column (3.9 × 300 mm), as described by San George and Hoberman [4]. Chromatography was carried out at 25°C at a flow rate of 1 mL/min, the eluent being monitored at 214 nm. Typically, 0.1–0.2 μmol of aqueous peptide/adduct was injected in a volume of 10–50 μL. The ratio of ϵ_{214} (adduct)/ϵ_{214} (peptide) was determined for each peptide by comparison of peptide peak areas in runs using peptide alone and adduct peak areas in runs using a mixture of peptide, and a large excess of AcH allowed to reach equilibrium. Blank experiments were also performed by chromatography of AcH solutions after incubation at 37°C in PBS, pH 7.4. Several small peaks and one major peak resulted from this incubation, but they did not interfere with measurements of peptide and adduct. Elution times for the peptides and their adducts were as follows: val-gly-gly, 4.7 and 6.6 min; phe-gly-gly, 11.8, 29.5, and 32.3 min (two adduct peaks; see Results); ala-gly-gly, 3.8 and 4.2 min; gly-gly-val, 5.8 and 8.5 min; gly-leu, 8.4, 12.8, and 14.3 min (two adduct peaks); and gly-leu-NH_2, 6.9, 12.3, and 14.2 min (two adduct peaks). The adduct elution times were identical in experiments covering the whole range of AcH (50 μM–500 mM) and peptide concentrations (2–50 mM) used. Peak areas were calculated by multiplication of peak height by width at half height or by cutting and weighing.

Peptide-adduct Formation

All reactions of peptides with AcH were carried out at 37 ± 0.2°C in PBS, pH 7.4. To prevent loss of AcH from the reaction mixtures, a series of identical mixtures in 500 μL plastic-capped centrifuge tubes sealed with Parafilm was prepared, with one tube for each time point. Before addition of AcH, all components of the reaction mixture were mixed and chilled. After addition of AcH, tubes were sealed and brought rapidly to 37°C. Routinely, the AcH concentration was determined (see below) before incubation at 37°C and after the last time point. Loss of AcH during the incubation was always ≤8%, in experiments where AcH was initially present in excess. AcH solutions were stored in sealed bottles at 4°C, and all pipettes and tips used for AcH solutions were prechilled.

Confirmation of Imidazolidinone Structure

50 mM solutions of val-gly-gly, ala-gly-gly, phe-gly-gly, and gly-gly-val in PBS, pH 7.4, were reacted with [1,2-^{13}C]AcH (500 mM) at 37°C for 10 min (total volume, 2 mL). Excess AcH was then removed by bubbling of nitrogen through the sample for 5 min at 25°C, and the adduct was purified by reverse phase HPLC.

The adduct peaks from the four peptides (including two separate adduct peaks for phe-gly-gly) were collected in glass tubes, and the pH was immediately adjusted to 7.4 with 0.1 M sodium hydroxide (NaOH). The adduct was lyophilized and redissolved in 1.5 mL of water and 1.5 mL of D_2O. The adduct samples and a sample of AcH in phosphate buffer (all in 50% v/v D_2O) were used in ^{13}C NMR spectroscopy. Samples were stored at −20°C for less than 24 hr before use.

An aliquot of each adduct solution used in NMR spectroscopy was subjected to HPLC. The peak area was determined by cutting and weighing, and compared to the peak area of a known amount of peptide. Taking into account the absorbance differences (ϵ_{214} adduct/ϵ_{214} peptide = 1.5), the adduct concentration could be calculated. This HPLC analysis also showed that the amount of degradation of adduct that occurred during preparation of samples for NMR was minimal.

NMR spectroscopy was performed by John Hanna at the Brisbane NMR Centre, Griffith University, Nathan, Queensland. All ^{13}C NMR spectra were run at ambient temperature on a Bruker CXP300 spectrometer operating at a ^{13}C frequency of 75.46 MHz. All spectra were obtained with a single pulse and acquire sequence covering a sweep width of 20 000 Hz with 16 K data points, P/4 pulse time of 8 μsec, and recycle time of 3 sec. Each free induction decay was treated with a line broadening factor of 1 Hz prior to Fourier transformation. The number of accumulations was 200 for AcH, val-gly-gly adduct, and ala-gly-gly adduct spectra, and 1100 for gly-gly-val and phe-gly-gly adducts.

AcH Assay

The concentration of AcH in stock solutions and reaction mixtures was determined spectrophotometrically at 340 nm

using alcohol dehydrogenase and NADH, by a method adapted from that of Bernt and Bergmeyer [13]. In a 3-mL quartz cuvette with a tightly fitting stopper were placed 2.0 mL of 0.25 M sodium phosphate buffer, pH 7.5, 200 μL of an NADH solution, and 20 μL of an alcohol dehydrogenase solution. The initial A_{340} was then measured. The NADH solution, prepared by dissolving 3.3 mg of NADH (disodium salt) and 40 mg of $NaHCO_3$ in 4 mL of distilled water, was stable for at least 14 days at 0–4°C. The alcohol dehydrogenase was made up as a 30 mg/mL suspension in a 60% saturated ammonium sulphate solution that was stable for several months at 0–4°C. Samples to be assayed were diluted in ice-cold water until they were 1–2 mM in AcH; and 50-μL aliquots of such solutions were added to the reaction mixture in the cuvette, which was then tightly stoppered and allowed to sit at room temperature until the reaction was complete (15 min). Under the conditions of the assay, the AcH is quantitatively converted to ethanol, so determination of the change in NADH concentration by the change in A_{340} (using $\epsilon^{NADH} = 6220\ M^{-1}cm^{-1}$) gives the AcH concentration.

Measurement of Peptide α-Amino Groups

TNBS reacts with primary amino groups to form a coloured product. The TNBS assay used was an adaptation of the method of Plapp *et al.* [14]. An aliquot of the peptide solution and enough water to adjust the aliquot volume to 300 μL were added to 1.5 mL of 0.2 M borate buffer, pH 9.5. A reference solution containing 1.5 mL of borate buffer and 300 μL of water was also prepared. Then, 150 μL of 7.2 mg/mL TNBS solution was added to both and, 15 min later, the absorbance at 367 nm of the peptide solution was read against the reference solution. The reaction time of 15 min was determined by following the increase in absorbance at 367 nm for the reaction of val-gly-gly with TNBS. Maximum colour development was apparent at 15 min and the absorbance started to decline after 20 min. A standard curve for the reaction of val-gly-gly with TNBS was determined and shown to be linear up to 40 μM val-gly-gly.

Stability Studies

The decomposition of the val-gly-gly adduct was studied by HPLC. To isolate the adduct, a reaction mixture containing 20 mM val-gly-gly and 200 mM AcH in PBS, pH 7.4, was incubated at 37°C for 24 hr. Then, serial injections of 100 μL were applied to the HPLC column and the adduct peak collected. Immediately, the pH of the adduct-containing fraction was adjusted to 7.4 using 1 M NaOH and the solution was placed on ice. Subsequently, the adduct solutions were stored at −20°C. HPLC analysis of the pooled adduct fractions revealed the concentration of the isolated adduct to be approximately 0.9 mM. It should be noted that the ionic strength of the phosphate buffer in which decomposition of the adduct was studied was 0.096, compared with 0.043 for the PBS. The adduct, at a concentration of ~0.75 mM, was incubated at 37°C and at 60°C, 80°C, and 100°C both in the presence and absence of 10 mM phe-gly-gly. The tubes were not tightly sealed, which allowed loss from the reaction mixture of AcH released by adduct breakdown. HPLC analyses were carried out at suitable time points. To compensate for evaporation, the tubes were weighed before each aliquot was taken and water added to restore the loss. This was never more than 10% of the volume of the reaction mixture.

Evaluation of First-order Rate Constants

The data were analysed by means of either infinity or Guggenheim plots. Both made use of the ratio F_t, the amount of peptide remaining (or adduct formed) divided by the original amount of peptide. F_∞ was the value of F_t obtained after at least 6 half-lives. In cases where it was impractical to obtain an infinity point (e.g. when the reaction was followed by the TNBS assay), a Guggenheim plot was used. Kinetic data are expressed as mean ± SD.

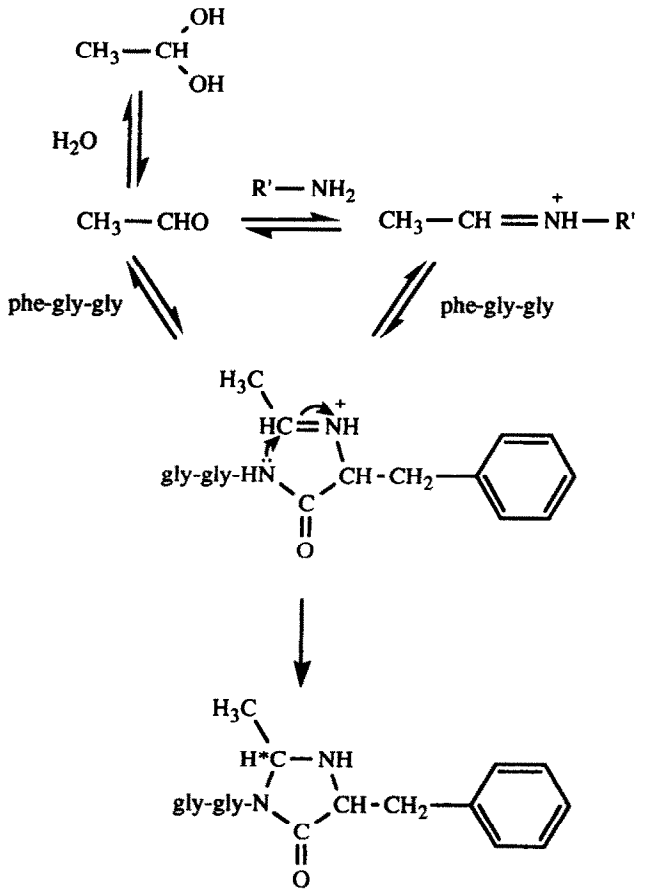
RESULTS

Evidence for the Imidazolidinone Structure of AcH-Peptide Adducts

Elution profiles for the reverse-phase HPLC of the mixture formed by reaction of phe-gly-gly with AcH showed a peak at 11.8 min, which represented unreacted peptide and new peaks eluting at 29.5 and 32.3 min. Based on the observations of San George and Hoberman [4] with val-gly-gly, it was proposed that these new peaks represented the diastereoisomeric forms of the 2-methylimidazolidin-4-one adduct of phe-gly-gly with AcH (Scheme 1). ^{13}C NMR spectra of these two adduct peaks, formed by reaction of phe-gly-gly with [1,2-^{13}C] AcH, were similar to those obtained for val-gly-gly, gly-gly-val, and ala-gly-gly adducts. Table 1 gives chemical shift data for the adduct resonances and the proposed assignments of these resonances. The doublets in the region of 18–22 ppm and those in the region of 72–74 ppm, present in all of the spectra, have been assigned to C-2 of the AcH moiety (the methyl carbon atom) and to C-1 of the AcH moiety, respectively. The latter assignment is reasonable for a carbon atom bonded to two electronegative atoms, as in the imidazolidinone structure [15]. San George and Hoberman [4] observed similar resonances for the adduct of val-gly-gly with AcH (Table 1). The 2-methylimidazolidin-4-one structure of peptide-AcH adducts with ^{13}C resonances and elution behaviour on reverse phase HPLC, as found in this study, has been strongly supported by mass spectroscopic studies in two laboratories [16, 17]. Further support for the imidazolidinone structure is provided by the two-dimensional proton NMR study of the adducts formed between AcH and enkephalins [7].

Reaction of val-gly-gly with AcH

Val-gly-gly (2 mM) was reacted with 5 different concentrations of AcH (17–87 mM) at pH 7.4, 37°C. In all cases,



SCHEME 1. Reactions occurring between AcH, phe-gly-gly and α-N-acetyl-L-lysine N-methylamide (R'-NH2). The pK'_a values of the α-amino group of phe-gly-gly and the ε-amino group of the lysine derivative are ~8 and ~10, respectively. The asterisk designates the new chiral carbon atom in the imidazolidinone adduct derived from the C-1 carbon of AcH.

the reaction went to completion (no residual peptide) and obeyed pseudo first-order kinetics. Figure 1 shows a typical infinity plot of the HPLC data and Table 2 lists the observed first-order rate constants (k_{obs}). A plot of k_{obs} against [AcH] (Fig. 2) was linear, with a slope of 0.734 ± 0.032 M^{-1} min^{-1}, which is the second-order rate constant k_2 for the reaction of val-gly-gly with AcH to form the imidazolidinone. Also in Table 2 are k_{obs} values for the same reaction at two AcH concentrations, followed by the TNBS method, measuring the loss of peptide. The satisfactory agreement between the rate constants determined by the different methods shows that loss of the α-amino group (measured by the TNBS method) and formation of the adduct (measured by HPLC) have the same rate-limiting step. The linearity of Fig. 2 shows that Schiff base formation prior to cyclization to form the imidazolidinone (Scheme 1) is not kinetically significant at concentrations of AcH up to 87 mM with a peptide concentration of 2 mM.

Val-gly-gly (20 mM) was next reacted with a series of concentrations of AcH such that the peptide was always in large excess. With $[AcH]_o$ = 1.82 mM, k_{obs} = 0.0166 min^{-1}, corresponding to a k_2 value of 0.83 M^{-1} min^{-1}. This value agrees reasonably well with the k_2 value determined in the presence of an excess of AcH (0.73 M^{-1} min^{-1}). Table 3 compares measured values for the ratio F_∞ with calculated values for F_∞, assuming that all of the AcH had been incorporated into adduct at equilibrium. The good agreement between measured and calculated values shows that the reaction yielding imidazolidinones proceeded to completion at all AcH concentrations used. These results with $[AcH]_o << [peptide]_o$ also confirm that the adduct formation observed by HPLC is due to the reaction of AcH itself with peptide and does not involve reaction with any impurity that might be present in the AcH solutions.

Reaction of Other Peptides with AcH

The reactions of three additional tripeptides and two dipeptides (all at 2 mM) with a large excess of AcH (52–61 mM) were followed by HPLC and values of k_{obs} were determined. The reactions with the tripeptides went essentially to completion (no residual peptide), but in the reactions of the dipeptides (gly-leu and gly-leu-NH_2), a significant amount of unmodified peptide (20% and 25%, respectively) remained at equilibrium. Values of k_{obs} and k_2 for each of the peptides are listed in Table 4.

Imidazolidinone Formation in the Presence of α-N-acetyl-L-lysine N-methylamide

The reaction of 2 mM phe-gly-gly with 0.23 mM AcH in the presence and absence of 10 mM α-N-acetyl-L-lysine N-methylamide was followed by HPLC. Progress curves for adduct formation are shown in Fig. 3, yielding (from infinity plots) k_{obs} values of 0.30 ± 0.01 hr^{-1} and 0.18 ± 0.01 hr^{-1} in the presence and absence of the lysine derivative.

Regeneration of Peptide from val-gly-gly Imidazolidinone

Incubation of the isolated val-gly-gly imidazolidinone at 37°C in PBS, pH 7.4, resulted in a very slow release of peptide (~10% in 4 days). Therefore, the decomposition of the adduct and concomitant regeneration of peptide were followed at higher temperatures (60°C, 80°C, and 100°C). The reaction proceeded to completion (no adduct remaining) and infinity plots were linear, giving the rate constants listed in Table 5. An Arrhenius plot using these values of k_{obs} was linear, and allowed an estimate to be made of k_{obs} at 37°C, *viz.* 0.0020 ± 0.0003 hr^{-1}, $t_{1/2}$ = 346 hr. The decomposition of the isolated val-gly-gly imidazolidinone was also studied at elevated temperatures in the presence of phe-gly-gly (10 mM). At each temperature, the value of k_{obs} in the presence of phe-gly-gly was significantly greater than in its absence (Table 5). HPLC analysis showed that some phe-gly-gly adduct was formed in this reaction, confirming that some transfer of AcH moiety had occurred.

TABLE 1. ^{13}C chemical shift data for peptide-AcH adducts

Peptide	δ ppm	Assignment*
phe-gly-gly (first adduct peak)	20.58, 21.74	C-2 diastereoisomer II
	73.29, 73.83	C-1 diastereoisomer II
phe-gly-gly (second adduct peak)	20.58, 21.72	C-2 diastereoisomer I
	72.64, 73.16	C-1 diastereoisomer I
val-gly-gly	20.82†, 21.34, 21.73 (18.90, 18.50)‡	C-2 of both diastereoisomers
	72.27, 72.79 (70.00)‡	C-1 diastereoisomer I
	73.94, 74.46 (71.70)‡	C-1 diastereoisomer II
gly-gly-val	20.54, 20.67	C-2 diastereoisomer I
	21.06, 21.11	C-2 diastereoisomer II
	74.60§	C-1 diastereoisomer I
	75.32§	C-1 diastereoisomer II
ala-gly-gly	20.29, 20.72	C-2 diastereoisomer I
	20.82, 21.24	C-2 diastereoisomer II
	72.52, 72.62	C-1 diastereoisomer I
	73.05, 73.15	C-1 diastereoisomer II

* Diastereoisomer I was defined as the isomer with the lowest chemical shift for C-1.

† Doublet unresolved for one of the diastereoisomers.

‡ Data of San George and Hoberman (1986); the reason for the consistent 2–3 ppm difference in chemical shifts between the two sets of results is not known.

§ Major peak.

DISCUSSION

The experiments reported here were performed as part of an investigation of the hypothesis that imidazolidinone adducts form and/or accumulate in the tissues of alcoholic patients. The results obtained will be discussed by attempting to relate the model system studied here to the *in vivo* situation. The tripeptides studied react readily with AcH to form adducts at physiological pH and temperature. The identification of these adducts as 2-methylimidazolidin-4-one derivatives of the peptides is based on their ^{13}C-NMR spectra and earlier studies, as discussed above. In the presence of an excess of either peptide or AcH, the reactions go to completion even at the lowest AcH concentration used (50 μM). The first question is whether or not the model

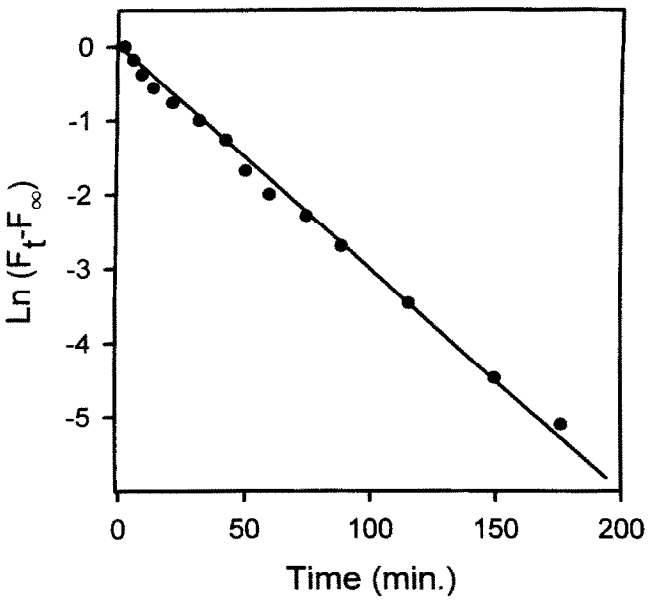


FIG. 1. Pseudo first-order plot for the reaction of val-gly-gly (2 mM) with AcH (36 mM) at pH 7.4, 37°C. The line is the linear least squares fit of the data, giving k_{obs} = 0.029 ± 0.001 min^{-1}.

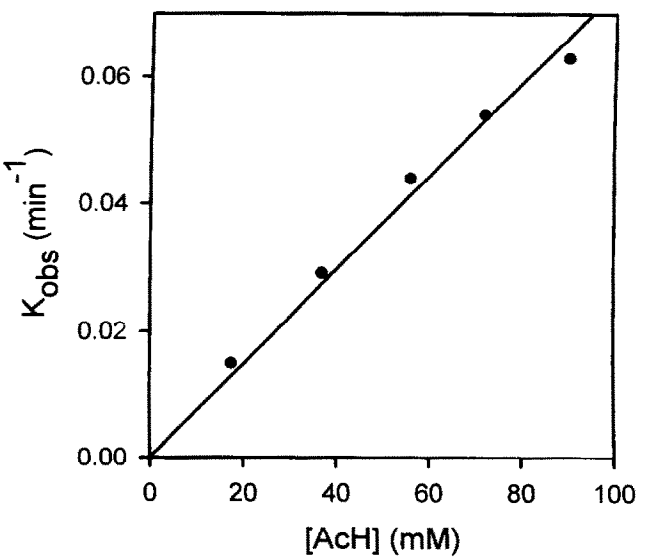


FIG. 2. Effect of AcH concentration on k_{obs} for the reaction of AcH (17–87 mM) with val-gly-gly (2 mM) at pH 7.4, 37°C. The linear least squares line gives k_2 = 0.734 ± 0.032 M^{-1} min^{-1}.

TABLE 2. Values of k_{obs} and $t_{1/2}$ for the reaction of 2 mM val-gly-gly with different concentrations of AcH at pH 7.4, 37°C

[AcH] (mM)	k_{obs} (min^{-1})	$t_{1/2}$ (min)
17	0.015 ± 0.001	46
17*	0.012 ± 0.001†	58
36	0.029 ± 0.001	24
54	0.044 ± 0.001	16
69	0.054 ± 0.003	13
83*	0.061 ± 0.001	11
87	0.063 ± 0.001	11

* Reactions followed by the TNBS method.
† Based on these values, a k_2 of 0.73 M^{-1} min^{-1} may be calculated.

TABLE 4. Values of k_{obs} and k_2 for the reaction of peptides (2 mM) with AcH at the concentrations specified at pH 7.4, 37°C

Peptide	[AcH] (mM)	k_{obs} (min^{-1})	k_2* (M^{-1} min^{-1})
phe-gly-gly	55	0.067	1.22 ± 0.07
ala-gly-gly	57	0.054	0.95 ± 0.02
val-gly-gly	54	0.044	0.734 ± 0.032†
gly-gly-val	61	0.048	0.79 ± 0.02
gly-leu-NH_2	62	0.079	1.52 ± 0.03
gly-leu	55	0.002	0.040 ± 0.001

* $k_2 = k_{obs}/[AcH]$.
† Calculated from Fig. 2.

peptides are comparable (in their reaction with AcH) with peptides and proteins present *in vivo*. The k_2 values for the four tripeptides examined (Table 4) differ by less than a factor of 2, showing that the side chain of the N-terminal residue is not very significant. The k_2 for the dipeptide amide gly-leu-NH_2 is similar to that for the tripeptides. However, the dipeptide gly-leu reacts much more slowly, as would be expected from the effect of the α-carboxylate ion on the nucleophilicity of the peptide bond nitrogen atom. The reaction of the dipeptides with AcH did not go to completion, in contrast to the reactions of the tripeptides under the same conditions. Summers [6] reported that the tripeptide tyr-gly-gly was the minimum portion of the enkephalin molecule needed for rapid adduct formation with AcH. The only previously reported rate constant for imidazolidinone formation from AcH and a peptide [11] is a k_{obs} of 0.05 min^{-1} for the pentapeptide, methionine-enkephalin, which converts to a k_2 of 0.69 M^{-1} min^{-1} (pH 7.0, 25°C). This is in good agreement with the values for the tripeptides and dipeptide amide in Table 4, even allowing for the different temperature; the small difference in pH is unlikely to affect the rate constant [9]. Therefore, the rate constant for imidazolidinone formation is not markedly affected by peptide chain length or sequence, and the values in Table 4 provide a reasonable estimate of k_2 for the reaction of protein α-amino groups with AcH. In polypeptides and proteins, the rate of imidazolidinone formation may, however, be affected by steric factors or involvement of the α-amino group in noncovalent bonds.

Using a second-order rate constant of 0.73 M^{-1} min^{-1} (at pH 7.4, 37°C) for the reaction of AcH with protein α-amino groups to form the imidazolidinone, it is possible to estimate the reaction rate *in vivo*. For example, the concentration of haemoglobin subunits in erythrocytes is about 20 mM, giving a k_{obs} of 0.014 min^{-1} ($t_{1/2}$ = 50 min). In liver cytosol, the concentration of protein α-amino groups is approximately 1.5 mM (based on a protein concentration of 100 mg/mL, an average subunit weight of 40 *k*Da and 50% of α-amino groups not being acetylated or otherwise unavailable for reaction [18]), giving a $t_{1/2}$ of about 10 hr for imidazolidinone formation. *In vivo*, the main competing reaction is metabolism of AcH in liver, catalysed by aldehyde dehydrogenase. The loss of AcH from blood after

TABLE 3. Observed and calculated values of F_∞ for the reaction of 20 mM val-gly-gly with different AcH concentrations at pH 7.4, 37°C

[AcH] (mM)	Calculated F_∞* (adduct)	Observed F_∞† (adduct)
0.91	0.046	0.05
0.46	0.023	0.024
0.16	0.008	0.0079
0.1	0.005	0.0053
0.05	0.0025	0.003

* Calculated F_∞ = the fraction of val-gly-gly converted to adduct assuming 100% conversion of the AcH to adduct.
† The fact that observed F_∞ values are, in most cases, greater than the values calculated for 100% reaction suggests that the peptide concentration was slightly less than 20 mM (e.g. because of impurities in the preparation of peptide used).

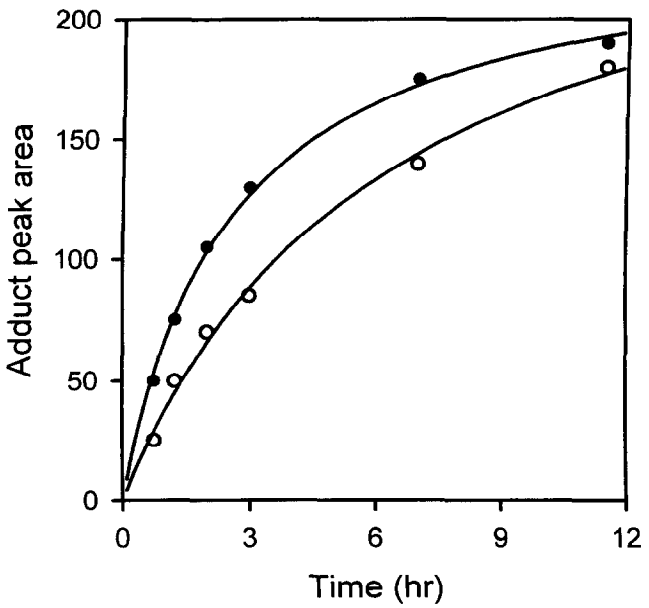


FIG. 3. Progress curves for the reaction of phe-gly-gly (2 mM) with AcH (0.23 mM) at pH 7.4, 37°C, in the presence (●) and in the absence (○) of α-N-acetyl-L-lysine N-methylamide (10 mM).

TABLE 5. Values of k_{obs} and $t_{1/2}$ for the decomposition of the val-gly-gly imidazolidinone (0.75 mM) at pH 7.4 at the temperatures indicated, in the presence and absence of phe-gly-gly

Temperature (°C)	k_{obs} (hr^{-1})	$t_{1/2}$ (hr)
60	0.038 ± 0.001	18.2
60*	0.060 ± 0.001	11.6
80	0.16 ± 0.005	4.3
80*	0.332 ± 0.004	2.1
100	0.71 ± 0.01	0.98
100*	2.73 ± 0.04	0.26

* Phe-gly-gly (10 mM) present in the reaction mixture.

cessation of alcohol ingestion occurs over a period of several hr [19]. Because the kinetics of adduct formation would be expected to be pseudo first-order in AcH concentration under *in vivo* conditions (since [α-amino-groups] >> [AcH]), the estimated $t_{1/2}$ is independent of the concentration of AcH. The results of Table 3 show that imidazolidinone formation goes to completion under conditions similar to those *in vivo*, at initial AcH concentrations down to 50 μM. In alcoholics, blood AcH concentrations reach 20–100 μM [19, 20] or even higher [21]. However, it should be noted that some researchers believe that some or all of the AcH detected in these, and other studies, may be due to artefactual generation of AcH from ethanol in the blood [22]. In the liver, the concentration in some cellular compartments during chronic ethanol metabolism may be 1 or 2 orders of magnitude higher than in blood [23]. Therefore, imidazolidinone formation is highly likely to occur in hepatocytes and, probably, also in the erythrocytes of people who ingest alcohol. The rate of formation of imidazolidinone in the erythrocytes of an alcoholic who maintained a blood AcH concentration of ~20 μM by continual drinking would be ~0.4 mM day^{-1} (0.014 min^{-1} × 20 μM). This suggests that a significant percentage of the haemoglobin could carry imidazolidinone adducts (0.4 mM = 2%).

In vivo, the concentration of ε-amino groups of proteins exceeds the concentration of α-amino groups by a factor of perhaps 10 (i.e. the average number of lysine residues per polypeptide chain). Therefore, the effect of α-N-acetyl-L-lysine N-methylamide (10 mM) on the kinetics of imidazolidinone formation from AcH (0.23 mM) and the tripeptide phe-gly-gly (2 mM) was determined, as a closer approximation to conditions *in vivo*. The lysine derivative caused an appreciable increase in k_{obs}. Scheme 1 shows the compounds likely to be present in this reaction mixture. To explain the rate acceleration caused by the lysine derivative, it is proposed that the Schiff base formed from AcH and the ε-amino group reacts with the α-amino group of the tripeptide more rapidly than does AcH, which is also present as the unreactive hydrate. Whatever the explanation, the data suggest that imidazolidinone formation *in vivo* would be facilitated, rather than hindered, by reaction of AcH with ε-amino groups of proteins to form unstable Schiff bases. The majority of AcH present in blood and liver during ethanol metabolism appears to be present in these linkages [20, 23]. However, other functional groups (particularly protein and nonprotein thiol groups) may also affect the rate and extent of imidazolidinone formation *in vivo* by forming adducts with AcH.

The present kinetic study, together with the findings of San George and Hoberman [4] that imidazolidinones are the major stable adducts formed from AcH and haemoglobin *in vitro*, strongly suggest that imidazolidinones do form in appreciable amounts *in vivo*. The next question is: to what extent will they accumulate *in vivo*? The only information on the stability of peptide-derived 2-methylimidazolidin-4-ones at physiological pH and temperature is that reported here: a $t_{1/2}$ of 346 hr (about 14 days) for the val-gly-gly derivative at pH 7.4, 37°C. This suggests that accumulation of imidazolidinone adducts occurs during periods of sustained drinking, but that they will decompose, regenerating the free α-amino group and AcH, over a period of several weeks after cessation of alcohol consumption. This picture is, however, complicated by the transfer reaction first reported by San George and Hoberman [4] and confirmed here, in which an imidazolidinone of one peptide can react with the α-amino group of a second peptide to form its imidazolidinone. This transfer reaction, therefore, competes with the decomposition of the imidazolidinone. Given the high concentration of protein α-amino groups *in vivo*, the transfer reaction is likely to result in a greater and more prolonged accumulation of imidazolidinone than would otherwise be the case. The extent of accumulation of imidazolidinone adducts of proteins also depends on the turnover rates of the proteins. Greater accumulation would be expected in the long-lived erythrocyte proteins than in the more rapidly metabolized hepatocyte proteins.

The results presented here show that a search for imidazolidinone adducts in the blood and liver of alcoholic patients is warranted. Several recent studies have shown the presence of AcH-modified proteins in the blood or liver of ethanol-fed animals and in human alcoholic patients [24–27]. However, the structures of these adducts remain to be established. Imidazolidinones are not the only adducts formed because actin [28] and calmodulin [29], both of which have blocked α-amino groups, form stable adducts when incubated with AcH *in vitro*. As shown by Israel *et al.* [30] and Worrall *et al.* [31], AcH-modified proteins can act as neoantigens, inducing an immune response that may result in some of the tissue damage caused by long-term alcohol abuse. Indeed, antibodies reactive with proteins modified by AcH *in vitro* and reduced by sodium cyanoborohydride (a reducing agent relatively specific for Schiff bases) have been detected in social and heavy drinkers, and in alcoholics [32–35]. In a previous study, we have shown that rats fed an ethanol-containing diet for prolonged periods generate antibodies reactive with proteins modified by AcH *in vitro* without reduction by sodium cyanoborohydride (conditions likely to generate imidazolidinone derivatives), suggesting that antibodies reactive with imidazolidinones may have been detected [36]. Gross and coworkers

have shown that incubating red blood cells or purified haemoglobin with AcH *in vitro* led to the generation of several types of adduct, including imidazolidinones at the N-termini of the α- and β-chains [37]. Furthermore, as mentioned above, AcH-modified haemoglobin has been detected in alcoholics by several groups [25, 38, 39]. Stockham and Blanke [17] were unable to detect the imidazolidinone adduct of the tryptic peptide βT-1 (1-8 of β chain) in samples of haemoglobin prepared from alcoholic patients. That study shows the technical difficulties involved in detecting a small amount of a specific modified haemoglobin. It will, therefore, be of interest to see whether or not antibodies against the imidazolidinone structure exist in alcoholic patients.

Much work is now being carried out to determine whether or not antibodies reactive with proteins modified by AcH *in vitro* can be used as markers of alcohol intake. In another study, we have shown that heavy drinkers and alcoholics exhibit elevated immunoglobulin A reactivity with AcH-modified proteins when compared to social drinkers [34]. However, the identity of the epitopes that these antibodies react with is, at present, unknown. Studies such as the one described in this report will allow a greater understanding of the types of adduct that may be generated *in vivo*. Furthermore, the delineation of conditions leading to the generation of known types of adduct will allow: 1. the measurement of antibody reactivity with known adduct structures, and 2. the generation of mono- and polyclonal antibodies against specific adduct types. This may, then, lead to a better diagnostic test for alcohol intake.

The assistance of Dianne Keough and Ian Tonks with some of the HPLC separations was greatly appreciated.

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