Pyruvic Acid Is Attached through Its Central Carbon Atom to the Amino Terminus of the Recombinant DNA-derived DNA-binding Protein Ner of Bacteriophage Mu*

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Ner protein of bacteriophage Mu, produced by recombinant DNA techniques in *Escherichia coli*, has been found to possess a molecule of pyruvic acid attached covalently through carbon-2 to the amino-terminal cysteine residue. The intact protein and the amino-terminal chymotryptic peptide were found by mass spectrometry to be 70 mass units heavier than expected. The modified peptide was unstable under mildly acid or mildly basic conditions. Two-dimensional nuclear magnetic resonance spectroscopy of the modified and unmodified forms of the amino-terminal chymotryptic peptide was consistent with the presence of pyruvate linked through carbon-2 to the amino-terminal Cys residue. Treatment of the modified form with 2,4-dinitrophenylhydrazine in acid medium led to the expected hydrazone of pyruvic acid, which was identified by high pressure liquid chromatography. Of the two proteins known to be modified by pyruvate through its central carbon (the other being human adult hemoglobin, in which the modified form represents only a very minor fraction), Ner is the first protein found to be modified quantitatively. Given the instability of the modification, it may be more prevalent than recognized hitherto. Incubation with 2,4-dinitrophenylhydrazine may offer a useful means of detecting the presence of pyruvate linked to proteins in this way.

The DNA-binding protein Ner of bacteriophage Mu is involved in the regulation, at the transcriptional level, of the choice between lysogenic and lytic development of the phage (1). Ner has been produced by recombinant DNA techniques in relatively large amounts in *Escherichia coli*, and characterized (2). *In vitro* and *in vivo* DNA binding properties of the plasmid-derived protein suggest that it has a similar conformation to the authentic Ner protein. Recombinant DNA-derived Ner was found to be a single component by electro-

phoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate and to be monomeric in the concentration range studied by sedimentation equilibrium centrifugation (0.05–0.31 mg/ml). Ner gave an amino acid analysis in excellent agreement with that predicted from the DNA sequence, and Edman degradation (2) identified residues 2–74 of this 74-residue protein. The amino-terminal residue, predicted to be Cys, was the only residue not positively identified by Edman degradation in this earlier work (2), but such a failure is not unusual with Cys residues which have not been alkylated prior to sequence analysis. The presence of this residue was demonstrated by amino acid analysis of the protein after oxidation of Cys to cysteic acid with performic acid.

Since the publication of the preparation and characterization of Ner, we have submitted the intact protein to analysis by electrospray ionization mass spectrometry. The molecule was found to be 70 mass units heavier than predicted for the determined sequence. This work is reported here, together with extensive further analysis which permits us to conclude that the amino-terminal residue of the protein is linked to a molecule of pyruvic acid through the central carbon atom of the latter.

Pyruvic acid has recently been found linked through its central carbon atom, via ketamine or enamine formation, to the amino-terminal valine of the β-chain of fraction A_{1b} of human adult hemoglobin (3). Extensive use of high performance mass spectrometry was required to determine the nature of the modification. The modification was found to be acid-labile, as is that of Ner reported here. Since the attachment of pyruvate through carbon-2 is labile and requires relatively sophisticated techniques to be identified, it may be more prevalent than the two examples known to date. Linkage through carbon-2 is not to be confused with the well known linkage through the carboxyl group (carbon-1) found in various decarboxylases and some reductases (4). In these latter cases, the pyruvoyl group plays an essential part in the activity of the enzymes in question.

MATERIALS AND METHODS

Unless otherwise specified, solvents and reagents were of analytical grade, were obtained from commercial sources, and were used without further purification.

Ner—The protein was prepared, purified, and characterized exactly as described previously (2). The buffer used for the final purification (100 mM Tris-HCl, pH 7.5, containing 2 mM NaN₃) was exchanged for 50 mM ammonium bicarbonate using a PD-10 gel filtration column (Pharmacia LKB Biotechnology Inc.). Portions were lyophilized for direct analysis by mass spectrometry.
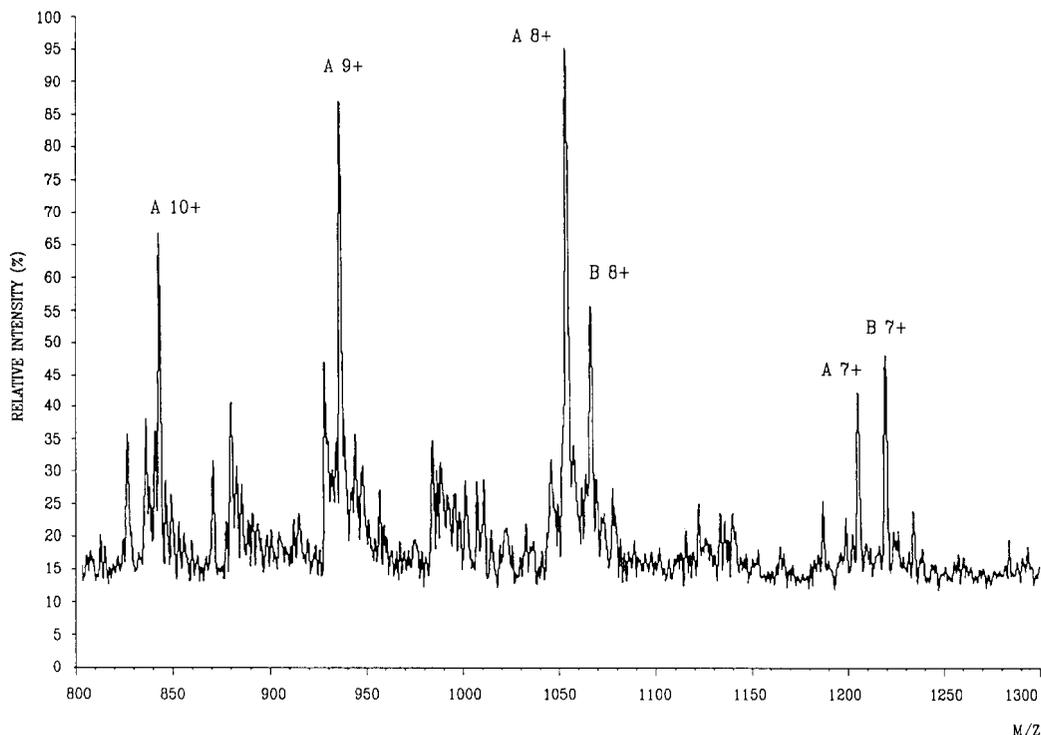

Pyruvic Acid Is Attached to Ner

FIG. 1. **Electrospray ionization mass spectrum of intact Ner.** This spectrum was acquired using a prototype VG Bio-Q instrument (VG BioTech, Altrincham, United Kingdom). The sample was initially dissolved in 5% aqueous acetic acid and then diluted with an equal volume of methanol. Ten μl of the resulting solution was introduced into the source at 3 μl/min and several scans summed over 5 min to obtain the final spectrum. The mass scale was calibrated with the multiply charged ion series from a separate introduction of horse heart myoglobin (Sigma; molecular weight 16,951.5). Two ion series are prominent. Series A leads to a mass assignment of 8,444.7 ± 1.0, and series B to a value of 8,542.3 ± 0.3.
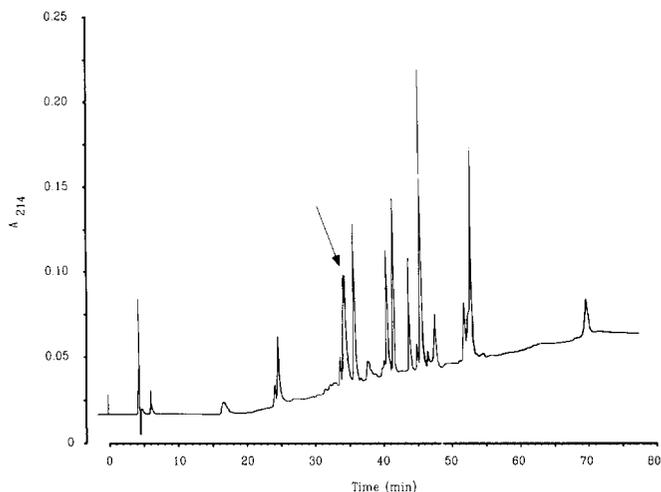

FIG. 2. **Analytical separation of the chymotryptic peptides of Ner.** Digestion with α-chymotrypsin (Sigma, enzyme:substrate ratio 0.015:1) was performed at a substrate concentration of 0.34 mg/ml in 50 mM ammonium bicarbonate, pH 7.8, at 37 °C for 90 min. Digests were lyophilized prior to injection onto HPLC. The sample (10 μg) was injected at 0% B, conditions which were maintained for 5 min, whereupon a linear gradient of 1% B/min was applied. Other chromatographic conditions were as decribed under "Materials and Methods." The modified amino-terminal 9-residue peptide was identified as the split peak *arrowed* in the figure and is referred to as species A.

High Pressure Liquid Chromatography—Reversed phase HPLC[1] was performed on Waters equipment. Solvent A was made by adding 1 g of trifluoroacetic acid (HPLC grade, Applied Biosystems Inc.) to 1 liter of HPLC grade water (MilliQ system) and degassing by vacuum filtration. Solvent B was made by adding 1 g of trifluoroacetic acid to 100 ml of degassed HPLC grade water then making up to 1 liter with degassed acetonitrile (gradient grade, Merck). A column 250 × 4 mm inner diameter packed with Nucleosil 5 μm 300 Å C8 (Machery Nagel) was used at a flow rate of 0.6 ml/min, monitoring at 214 nm for peptides and at 214 or 365 nm when analyzing 2,4-dinitrophenylhydrazine derivatives. Components were collected manually at the detector outlet and solvents removed in a vacuum centrifuge (SpeedVac, Savant) without heating.

RESULTS AND DISCUSSION

Intact Protein—Analysis of intact Ner by electrospray mass spectrometry gave rise to two major ion series (Fig. 1). The A series is caused by multiple protonation of a species of molecular weight of 8,444.7 (±1.0). The B series is similarly caused by a species of molecular weight of 8,542.3 (±0.3). The A series is greater by 70.2 (±1) atomic mass units than the corresponding value (8,374.5) calculated from the known sequence (2), and the B series is greater than the A series by 97.6 (±1.3) atomic mass units. Noncovalent adducts of 98 atomic mass units (sulfuric or phosphoric acid) are commonly found to be associated with proteins (5) and are probably caused by ammonium sulfate used during protein isolation. From this analysis of the intact protein, we are led to conclude from the A series of ions that Ner is too heavy by 70.2 (±1) atomic mass units, but the association with an extraneous molecule could be noncovalent or covalent. We therefore decided to map the chymotryptic peptides of Ner in an at-

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[1] The abbreviation used is: HPLC, high pressure liquid chromatography.

TABLE I

Results of amino acid analysis of both forms of the amino-terminal chymotryptic peptide (residues 1–9)

Peptides were hydrolyzed at 150 °C for 20 min in a mixture of 12 M HCl and trifluoroacetic acid (2:1, v/v), and the resulting amino acids were analyzed on a Beckmann 6300 automatic analyzer (ninhydrin system).

Residue	Theoretical[a]	Species A		Species B	
		Direct[b]	Performic[c]	Direct[b]	Performic[c]
Asx	2	1.98	2.22	1.98	2.04
Ser	1	1.44	1.30	1.05	1.04
Glx	1	1.03	0.94	0.99	0.98
Ala	1	1.58	1.49	1.02	1.05
Lys	1	0.98	0.96	1.02	0.96
Arg	1	1.02	0.96	0.99	1.00
Cys	1	0.13	0.92	0.18	0.91
Trp	1	ND[d]	ND	ND	ND

[a] Theoretical composition based on sequence data.
[b] Direct hydrolysis.
[c] Hydrolysis was preceded by oxidation with performic acid.
[d] ND, not determined.

tempt to localize the possible presence of a modifying group. The amino terminus was suspected inasmuch as the amino-terminal Cys residue, although it yielded cysteic acid upon oxidation with performic acid, was the only one not positively identified by Edman degradation (2).

Digestion with Chymotrypsin—An analytical separation of the chymotryptic peptides is shown in Fig. 2. The doublet indicated by an *arrow* was identified as the amino-terminal peptide. Residues 1–8 were clearly identified by amino acid analysis of this fraction (Table I), and the presence of Trp (residue 9, the chymotryptic cleavage point) was confirmed by fast atom bombardment mass spectrometry (see below).

Stability of the Amino-terminal Peptide—The amino-terminal chymotryptic peptide as originally isolated is a doublet (Fig. 2) and is referred to as species A. Incubation of species A at pH 7.8 in ammonium bicarbonate solution led to formation of a later-eluting component referred to as species B (Fig. 3A). Preparative separation of the two components was achieved. Species B was found to be perfectly stable to incubation either in 0.1% trifluoroacetic acid or in 0.1 M phosphate, pH 7.0, for at least 6 h at room temperature (not shown). Species A, which was somewhat unstable in ammonium bicarbonate (see above), was quite unstable in 0.2% trifluoroacetic acid, and over a period of hours species A was found to decompose to give two earlier-eluting components, the earlier of which predominated at longer times (Fig. 3B). Species A was, however, found to be stable for at least 4 h 30 min at room temperature in 0.1 M phosphate at pH 7.0, which were the conditions used during the NMR experiments.

Mass Spectrometry of the Amino-terminal Peptide—Negative ion fast atom bombardment mass spectrometry (Fig. 4) of the amino-terminal chymotryptic peptide as isolated (species A) showed a signal at m/z 1,176.5, which is 70 mass units heavier than predicted (1,106.468) for the deprotonated monoisotopic molecular ion of residues 1–9 in unmodified form. Species B produced a signal at m/z 1,106.5 (*inset* to Fig. 4), a mass which corresponds to that predicted for the unmodified peptide. It is thus reasonable to conclude that a covalent modification of the amino-terminal peptide is lost from a proportion of the peptide during incubation in ammonium bicarbonate.

NMR Spectroscopy of the Amino-terminal Peptide—Fig. 5 shows the HOHAHA spectra for both the modified peptide (species A, Fig. 5A) and the unmodified peptide (species B, Fig. 5B). As is clearly evident, all amino acid spin systems for CSNEKARDW are observed in Fig. 5B, and the chemical
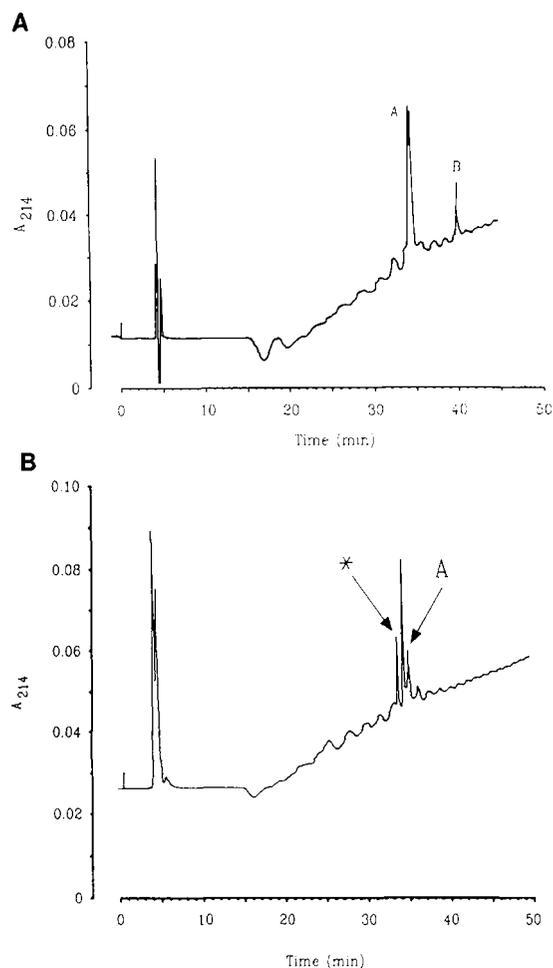
A

B

FIG. 3. **Stability of the amino-terminal chymotryptic peptide.** The amino-terminal chymotryptic peptide was incubated in 50 mM ammonium bicarbonate, pH 7.8, at room temperature (about 23 °C). After 2 h the sample was diluted with solvent A and subjected to HPLC. Chromatographic conditions were as in Fig. 2. The component eluting with unchanged retention time (species A) and a later-eluting component (species B) formed during the incubation were collected and dried. Portions of both components were incubated separately under various conditions for various times at room temperature, then injected onto HPLC. *Panel A*, species A after 2 h in 50 mM ammonium bicarbonate; the later-eluting species is the species B formed. *Panel B*, species A after 4 h in 0.2% trifluoroacetic acid; the *asterisk* denotes the component which predominates after 24 h.

shifts are very close to the random coil shifts, indicating that the peptide is essentially unstructured in solution. Comparison with Fig. 5A reveals a striking difference. The cross-peaks corresponding to the $C^{\alpha}H/C^{\beta}H$ correlation for Cys-1 are shifted and split into two sets of resonances, whereas all other cross-peaks remain essentially unchanged. In addition, resonances for two sets of methyl groups are observed. These are marked by *arrows* in the one-dimensional spectrum. The spin system marked C-1′ is of lower intensity than that of the major form C-1 and constitutes approximately 30% of the major form; there is no indication as to interconversion of these forms on the NMR time scale. These findings indicate that only Cys-1 carries a modification, given that chemical shifts are extremely sensitive to any change in the vicinity of the observed resonance, and no changes are observed for any of the other resonances. Evidence for the structure of this modification is discussed below.

Nature of the Modifying Group—The recombinant-derived Ner protein is associated with a species (or species) leading to a mass increment of 70 atomic mass units (electrospray
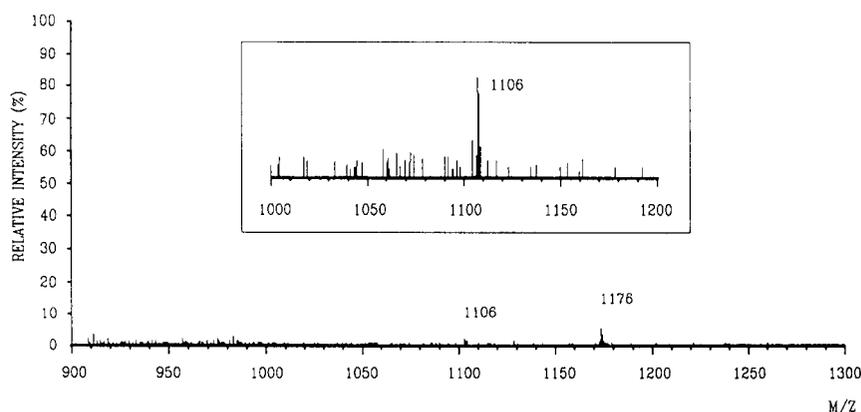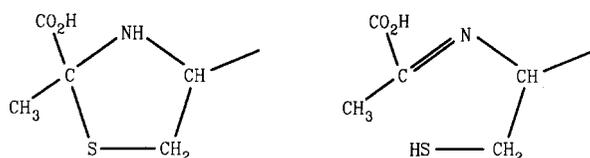
FIG. 4. **Mass spectra of the amino-terminal chymotryptic peptide, species A and B.** Fast atom bombardment mass spectrometry was performed on an MS 50 S (Kratos) equipped with a full FAB retrofit (M-Scan Ltd.) and operated under control of a DS 55M data system (Kratos). The machine was used in negative ion mode at an accelerating voltage of 4 kV with 1-aminopropane-2,3-diol as matrix (6) at a resolution of about 2,500 and was scanned at 30 s/decade. *Main spectrum* is of species A; *inset* is that of species B.

SCHEME 1. **Proposed structures of the modified amino-terminal residue.**

data obtained with intact Ner). This species is associated with the amino-terminal chymotryptic peptide of 9 residues (fast atom bombardment mass spectrometry) and is located on the amino-terminal residue (NMR).

The NMR results suggest that species A carries a molecule of pyruvate linked to the amino-terminal Cys residue. From mass spectrometry, we know that the attachment involves loss of water since the mass difference found is only 70 (the molecular weight of pyruvic acid is 88). The linkage between pyruvic acid and the peptide is not via an amide, ester, or thioester bond since a methyl group attached to a carbonyl group such as CH_3-CO-CO- would resonate downfield of 2 ppm, whereas we observe the methyl resonances of the modifying group at 1.7 ppm. This leads us to conclude, on the basis of the NMR evidence, that linkage is via carbon-2 (see Scheme 1). Both of the structures shown in Scheme 1 are compatible with the NMR data, and both offer an explanation for the existence of two sets of resonances for the methyl protons (and for the peak splitting seen on HPLC, Fig. 2): the imine structure would exist as syn and anti isomers about the C=N bond, and the cyclic structure would exist as two diastereoisomeric forms because of the asymmetry of the central carbon of the reacted pyruvate residue. Alternatively, the imine and cyclic structures could be responsible for the two sets of C-1 resonances (and the peak splitting seen on HPLC) and no resolution obtained between the geometrical isomeric forms of the two structures. Chemical evidence, in the form of marked instability to mild acid treatment, is also in accord with the structures shown in Scheme 1 and rules out an *N*-pyruvoyl structure which is very stable. When pyruvate is linked via carbon-1 (*i.e.* by an amide or ester bond) harsh treatment (1.5 M HCl, 100 °C, 1.5 h, or 0.1 M NaOH, 25 °C, 1-2 h, respectively) is necessary to free it from protein (4 and refs. cited therein). Since the imine structure shown in Scheme 1 possesses a free thiol group whereas the cyclic structure does not, an alkylation experiment should in principle permit the two structures to be distinguished. In-

cubation of both forms of the amino-terminal peptide with 10 mM dithiothreitol (1 h at 37 °C) followed by 30 mM vinylpyridine in 0.1 M ammonium bicarbonate, pH 7.8, for 30 min at room temperature led to quantitative alkylation of species B (as determined by HPLC and fast atom bombardment mass spectrometry), whereas very little alkylation of species A occurred under these conditions (not shown). The small amount of alkylation observed with species A is consistent with its slow decomposition (which occurs in ammonium bicarbonate; see Fig. 3A) into species B, which alkylates readily. In a similar experiment carried out in phosphate buffer at pH 7.0 (conditions under which species A is stable) and using iodoacetate in place of vinylpyridine, species B was found to be alkylated quantitatively within 15 min, whereas species A was not alkylated even after 100 min (not shown). These results suggest that the thiol group of the modified peptide (species A) is not readily available and would favor the cyclic structure over the imine. In the case of hemoglobin fraction A_{1b} (3), a cyclic structure is not possible since the amino-terminal residue is Val. Whichever of the two structures shown in Scheme 1 is correct, pyruvate is attached through carbon-2 to the amino-terminal Cys residue.

Confirmation of the Nature of the Modifying Group—To obtain a positive identification of the modifying group, we incubated intact Ner protein, both forms (species A and B) of the amino-terminal chymotryptic peptide, a pyruvic acid standard and controls, with 2,4-dinitrophenylhydrazine.

The amino-terminal peptide species A gave, on incubation with reagent, evidence of formation pyruvate 2,4-dinitrophenylhydrazone (Fig. 6). In this figure, peaks labeled with an *asterisk* are present in reagent blanks; peak 1 corresponds to species A decomposed by the acid reagent solution; peak 2 corresponds to the small quantity of species B which was present in the sample used; and peak 3 elutes at the same retention time as the principal isomer of a standard of the 2,4-dinitrophenylhydrazone of pyruvic acid. Species B was not affected under these reaction conditions and eluted unchanged as peak 2 (not shown). Since mass spectrometry had shown species B to be the unmodified peptide, we did not expect any hydrazone to be produced in this case.

To confirm the above finding in the case of intact Ner protein and to examine in a preliminary way the utility of hydrazone formation to detect pyruvate in proteins, we incubated Ner and two control proteins, α-lactalbumin and soybean trypsin inhibitor, with 2,4-dinitrophenylhydrazine. To avoid intense protein absorbance at 214 nm, we switched to
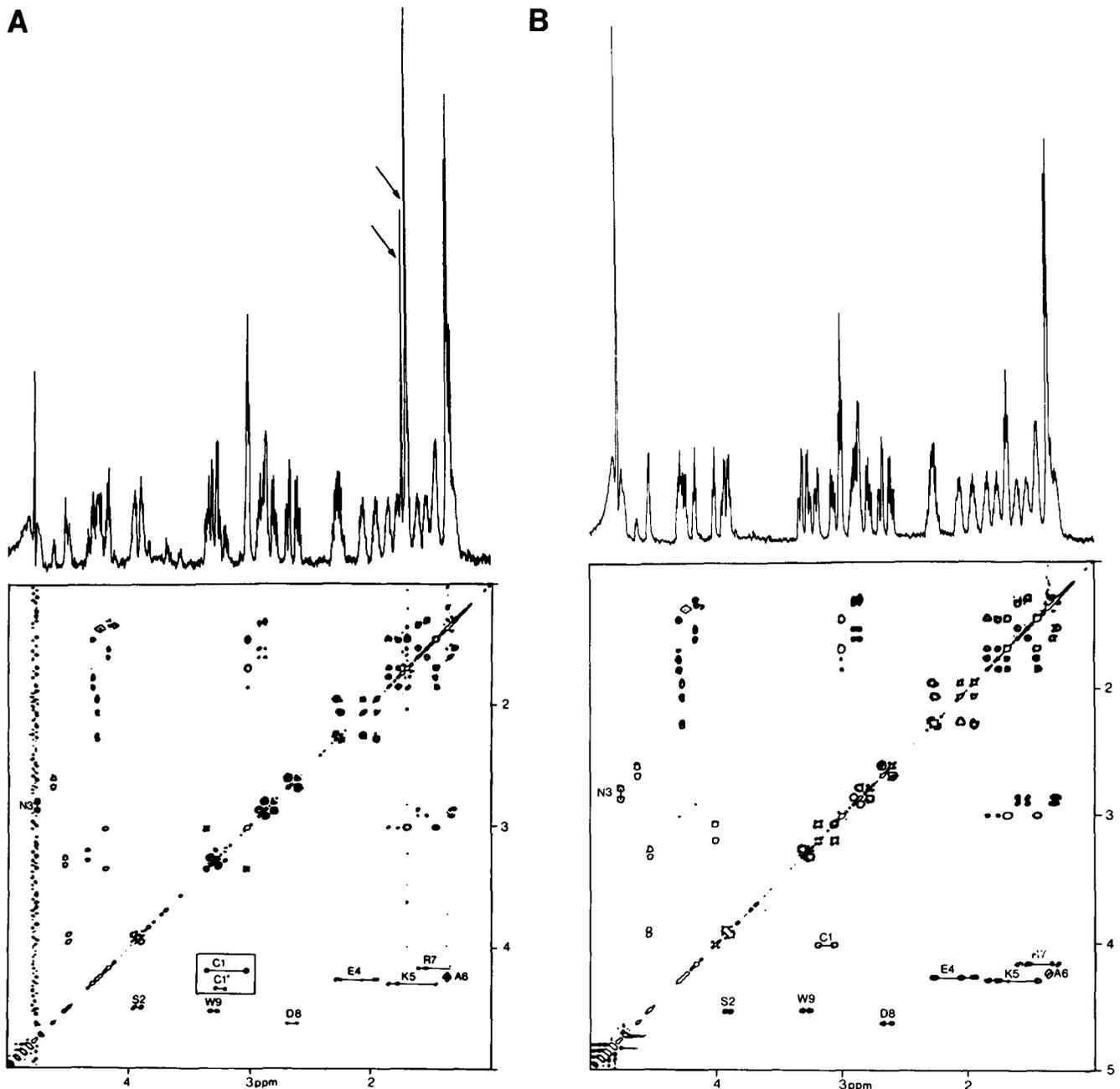
A   **B**

FIG. 5. **NMR spectra of the amino-terminal chymotryptic peptide, species A and B.** Spectra were obtained as described under "Materials and Methods." *Panel A*, species A; *panel B*, species B. Samples for NMR spectroscopy contained approximately 80 μM peptide in D_2O containing 50 mM phosphate buffer, pH 7.0. Spectra were recorded on a Bruker AM600 600 MHz spectrometer at 25 °C. Pure phase absorption HOHAHA experiments employed a Waltz 17 mixing sequence (7) with a total mixing time of 38 ms. 512 t_1 increments of 2,000 data points were collected and processed by appropriate zero filling to give a digital resolution of 4 Hz/point in F_1 and F_2. Chemical shifts are expressed relative to 4,4-dimethyl-4-silapentane-1-sulfonate.

365 nm to detect the hydrazone (we also shortened the analysis time by starting the chromatography at 20% B). Fig. 7, *trace A*, shows the chromatogram obtained on injection of 100 μl of a reaction mixture of equal volumes of pyruvic acid (0.01 nmol/μl) and reagent. The peak *arrowed* was thus obtained with the equivalent of 0.5 nmol of pyruvate injected (and would correspond to this amount if hydrazone formation was quantitative). Fig. 7, *trace B*, shows the result obtained with 1.15 nmol of Ner. Comparison with *trace A* and assumption of quantitative reaction in both cases lead to an estimate of

1.16 mol of pyruvate/mol of Ner. Fig. 7, *trace C*, was obtained with a reagent blank, and *traces D* and *E* with the control proteins, α-lactalbumin and soybean trypsin inhibitor, respectively. With the control proteins, no excess absorbance over the reagent blank is noted at the retention time of the pyruvate hydrazone. Incubation with 2,4-dinitrophenylhydrazine and detection of the liberated dinitrophenylhydrazone would thus seem to be useful for detecting pyruvate linked through carbon-2 to protein, although more work would be required to establish it as a reliable, quantitative method.
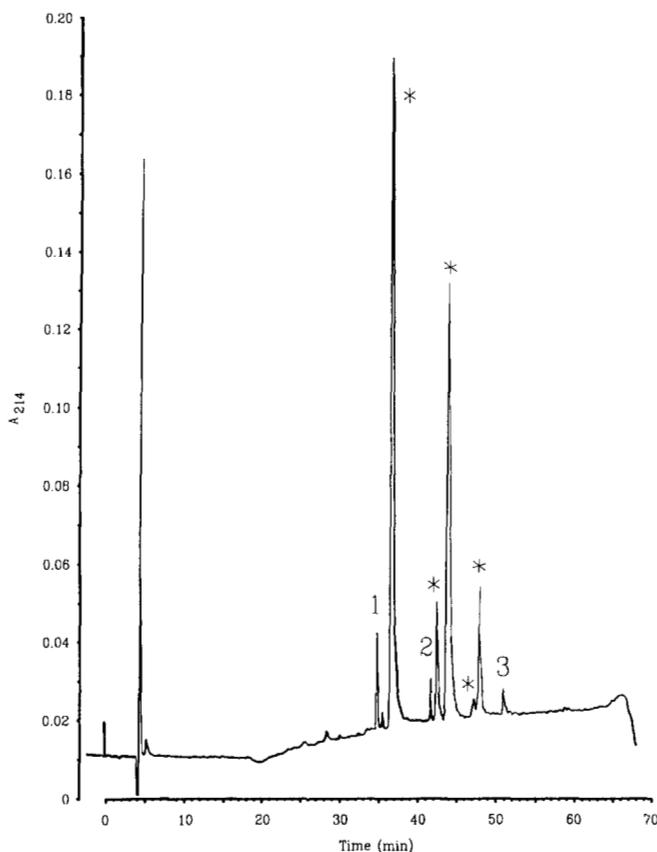
FIG. 6. **Analysis of the reaction of species A with 2,4-dinitrophenylhydrazine.** A solution of species A (about 0.5 mM in 50 mM phosphate, pH 7) was mixed with an equal volume of reagent (2,4-dinitrophenylhydrazine, about 17 mM, in 2 M HCl). After 1 h at room temperature, 2 μl was diluted with 50 μl of 0.1% trifluoroacetic acid, and 40 μl of the diluted reaction mixture was injected onto HPLC at 0% B, conditions which were maintained for 5 min, whereupon a linear gradient of 1% B/min was applied to 50% B, then 10% B/min to 100% B. A Kratos 757 detector was used with a 1-mm path length cell. Other chromatographic conditions were as described under "Materials and Methods." Peaks labeled with an *asterisk* were present in reagent blanks. Peaks 1–3 are discussed under "Results and Discussion."

Proteins possessing pyruvate linked through carbon-1 require harsh conditions to liberate pyruvate (see above), so such cases should not pose a problem.

Of the two proteins known to be modified by pyruvate through its central carbon (the other being human adult hemoglobin, where the modified form represents only a very minor fraction), Ner is the first protein found to be modified quantitatively. Given the instability of the modification, it may be more prevalent than recognized hitherto. Pyruvate is a prosthetic group of functional significance when attached to protein through carbon-1 in an amide or thioester bond (4). Determining the state of the amino terminus of natural (nonrecombinant, and so not over expressed) Ner, and whether its possible modification by pyruvate through carbon-2 has any functional significance, is an objective of future work.
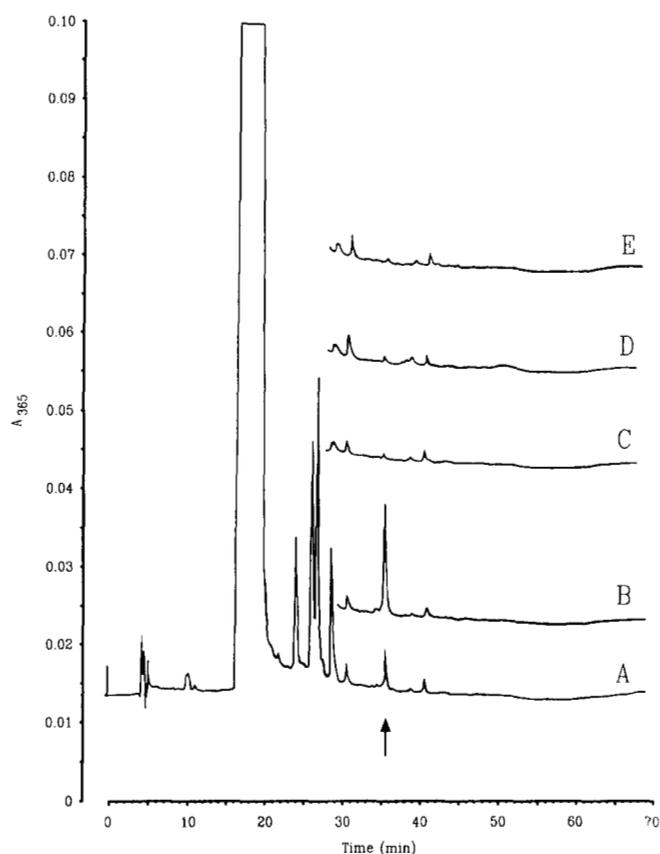

FIG. 7. **Analysis of reactions of proteins with 2,4-dinitrophenylhydrazine.** A solution of protein, 0.023 mM in 50 mM ammonium bicarbonate, was mixed with an equal volume of 2,4-dinitrophenylhydrazine (about 1 mM in 2 M HCl). Samples of 50 mM ammonium bicarbonate and 0.01 mM pyruvic acid were treated similarly. After 3 h at room temperature, 100 μl was injected onto HPLC. A Waters 490E detector was used with a 1-cm path length. Samples were injected at 20% B, conditions which were maintained for 5 min, whereupon a linear gradient of 1.25% B/min was applied for 30 min. Other chromatographic conditions were as described under "Materials and Methods." The *arrow* indicates the elution position of the 2,4-dinitrophenylhydrazone of pyruvic acid. Reactions with *A*, pyruvate 0.01 nmol/μl; *B*, Ner; *C*, reagent blank; *D*, α-lactalbumin (Sigma, product L-6385); *E*, soybean trypsin inhibitor (Sigma, product T-9003).

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