CHARACTERIZATION OF THE CARBAMINO ADDUCTS OF INSULIN

RICHARD H. GRIFFEY,* MARINA SCAVINI,‡ AND R. PHILIP EATON‡
*Center for Non-Invasive Diagnosis and ‡Department of Medicine, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

ABSTRACT Carbon-13 (^{13}C) nuclear magnetic resonance spectroscopy (NMR) is performed to characterize the formation of carbamino adducts between insulin and (^{13}C) carbon dioxide over a range of pH values in the presence of a physiological concentration (23 mM) of sodium bicarbonate. The peaks from two of the carbamino adducts resonate at higher frequencies than the signal from bicarbonate, at 164.6 and 165.3 ppm, and are attributed to the adducts with the terminal amino groups of phenylalanine B_1 and glycine A_1. The intensities of these signals vary with the pH, with unique patterns. Over 6% of each terminal amino group exists as the carbamino adduct at the optimum pH values of 7.8 and 8.3. A unique third adduct resonates at 159.3 ppm, and is attributed to lysine B_{29}. This adduct is present on 2% of the insulin molecules at pH 8.2, but has minimal intensity at pH 7.4. No signals from adducts are detected below pH 6.2, where the amino groups exist predominately in the protonated form. Creation of the adducts is rapid and they are stable for over 4 wk at 37°C. The narrow bandwidth of the resonance of the adduct (4.0–4.5 Hz) relative to the irreversible cyanate adduct is consistent with molecular forms of the carbamino adduct smaller than the 2-Zn-hexamer which is the preponderate form of clinically utilized U-100 insulin (i.e., 100 U/ml).

INTRODUCTION

U-100 insulin exists primarily in the 2-Zn-hexamer molecular form, in equilibrium with small amounts of the tetramer, dimer, and monomeric structures. The chemical properties of the three amino groups of insulin, Gly A1, Phe B1, and Lys B29, are functionally important to the surface interactions and self-association phenomenon of the molecule (1, 2). Above physiological pH, a conformational state of increased association of insulin solutions has been reported with a marked reduction in the chemical reactivity of the Gly amino terminus and the Lys group, with the Phe amino terminus remaining chemically reactive. At pH 7.0 to 7.3, the reaction profiles of the amino groups of Gly and Phe exhibit a marked increase, interpreted as dissociation to the monomeric form of insulin, with deprotonation of their amino groups (2). Acetylation of all three primary amino groups has no effect on the biological activity of insulin, so that the importance of their chemical properties applies predominately to the biological stability of insulin preparations (3).

Investigations conducted in the presence of bicarbonate at physiological pH in vivo have observed improved stability of insulin solutions (4, 5). The explanation for this observation has not been established, but is consistent with formation of carbamino adducts at the amino groups of insulin. Carbamino adducts have been detected and quantitated using ^{13}C nuclear magnetic resonance (NMR) spectroscopy over physiologically important ranges of pH and pCO_2 for several peptides of biological interest, including hemoglobin, glucagon, bradykinin, and angiotensin II (6–8). It has been previously suggested (6–8) that any peptide can be expected to form such an adduct with CO_2 unless its amino groups are chemically or structurally unavailable, and the possibility of such a reaction with insulin is attractive to explain the improved stability in the presence of bicarbonate.

The present study investigates the formation of carbamino adducts between U-100 insulin (0.75 mM) and (^{13}C) bicarbonate at clinically relevant concentrations over a range of pH between 6.2 and 8.6 in the absence and presence of zinc.

MATERIALS AND METHODS

Lyophilized sodium pork insulin was obtained from Eli Lilly & Co., Indianapolis, IN (CT 5706), and diluted to a strength of U-100 with isotonic saline. Zinc human insulin (U-100) was a generous gift from the Hoechst Corporation (HOE 21 PH, Frankfurt, FRG). This human insulin is in a solution at pH 7.0 with Tris-(hydroxymethyl) amino methane containing 10 μg/ml of polyethylene polypropylene glycol, 28 μg/ml of Zn^{++}, and phenol as a bacteriostatic agent. Zinc pork insulin was obtained from Eli Lilly & Co. (CT 5704). This porcine insulin is an aqueous solution of purified pork zinc-insulin crystals containing 1.0% glycerin, 0.25% in cresol as a preservative, and adjusted to neutral pH with NaOH and HCl during manufacture. Sodium (^{13}C) bicarbonate (99% ^{13}C) was purchased from Cambridge Isotope Laboratories, Woburn, MA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, and were of the highest available purity.

All samples were degassed with nitrogen before NMR experiments. Carbon-13 NMR spectra were obtained on a 7-tesla General Electric

Please address correspondence to R. Philip Eaton, M.D., University of New Mexico, School of Medicine, Department of Medicine, Albuquerque, NM 87131.

GN-300 instrument operating at a frequency of 75.82 MHz. A pulse repetition of 6 s and a 45° tip angle were employed to avoid saturation. Broadband proton decoupling was accomplished using a Waltz-16 sequence supplied by the manufacturer. Typically, a total of 8,000–12,000 transients were summed, multiplied by an exponential function corresponding to 8 Hz line broadening, and transformed. All chemical shifts were measured relative to dioxane and are expressed relative to tetramethylsilane. Circular dichroism (CD) data were obtained on a Jasco 40 instrument. NMR samples were diluted to 0.2 OD units at 280 nm in 50 μM Tris buffer (sodium chloride, 140 mM; pH 7.4) for these CD studies.

RESULTS

Carbon-13 Nuclear Magnetic Resonance Spectroscopy

The addition of 23 mM sodium (^{13}C) bicarbonate to a solution of zinc human insulin yields five new resonances in the ^{13}C NMR spectrum of the protein. As shown in Fig. 1, bicarbonate produces a signal at 161.2 ppm. The relative intensity of this signal and the signal from carbon dioxide at 126.2 ppm vary from 99:1 at pH 8.6 to nearly 1:1 at pH 6.2. Three other new signals are detected between pH 6.2 and pH 8.6. These signals, labeled A, B, and C, appear at 165.3, 163.6, and 159.3 ppm. They are assigned to the carbamino adducts which form between (^{13}C) carbon dioxide and insulin in the specified range of pH. A component of peak C is made up of the bicarbonate adduct of the Tris buffer, which has a chemical shift sensitive to pH. A fourth peak, D, in Fig. 1 is present in the absence of sodium bicarbonate and carbon dioxide in degassed solutions, and is generated from the natural abundance of ^{13}C in amide groups of the insulin.

The mole fraction of each amino group on the insulin molecules present as the carbamino adduct can be calculated from the known concentration of bicarbonate, carbon dioxide, protein, and adduct as described by Gurd and co-workers (7, 8). Fig. 2 illustrate the amount of adduct observed for each of the three carbamino peaks as a function of the pH of the insulin solution. The proportion of adduct B increases from pH 6.2 to pH 7.7, and declines from pH 7.7 to pH 8.6. In contrast, the proportion of adduct A increases over the range from pH 6.7 to pH 8.3, but the amount of adduct A is less than the amount of adduct B up to pH 8.6. The third peak, C, increases in intensity much more slowly, to a constant level between pH 7.7 and pH 8.6. As shown in Fig. 2, we calculate that 4–5% of U-100 insulin has formed the adduct B, 3–4% the adduct A, and 1–2% the adduct C at pH 7.4 in the presence of 23 mM sodium bicarbonate. The stability of these adducts was monitored at weekly intervals. No change in the ^{13}C NMR spectrum was noted after 4 wk in sealed glass NMR tubes at 37°C.

Based upon the pKa of Phe, Gly, and Lys, the equations of Gurd and co-workers can also be used to generate putative curves for the theoretical amount of adduct which would be formed as a function of the pH, bicarbonate concentration, and pCO_2. The pKa for the amino group of Phe-B1 has been determined to be 7.2 ± 0.2 from acetylation studies, whereas the pKa for the amino group of Gly-A1 has been determined to be 7.8 and for Lys-B29 to be 8.5 using the same method. Fig. 3 shows the expected concentration of bicarbonate adduct the Phe-B1, Gly-A1, and Lys-B29 over a range of pH from 6.0 to 8.5. The shapes of these curves agree well with the observed concentrations of adduct in peaks B, A, and C determined from the NMR measurements (see Fig. 2).

The linewidths of the signals range from 4.0 to 4.5 Hz over the range of pH studied. Whereas some of this

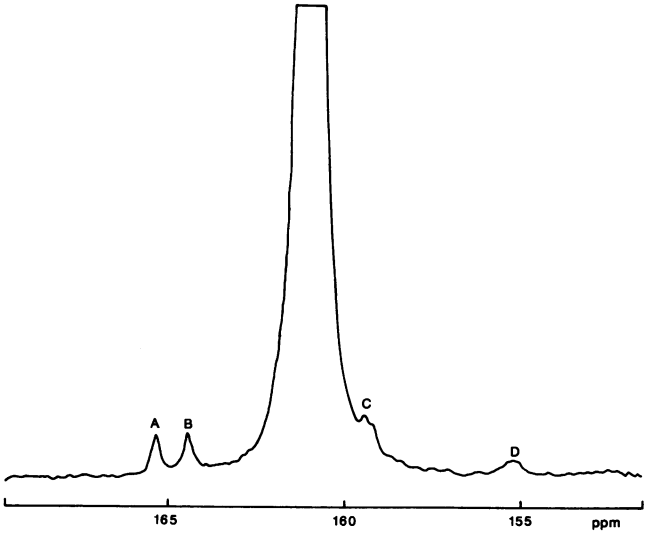

FIGURE 1 Carbon-13 NMR spectrum of human zinc insulin in an aqueous solution of 23 mM sodium bicarbonate.

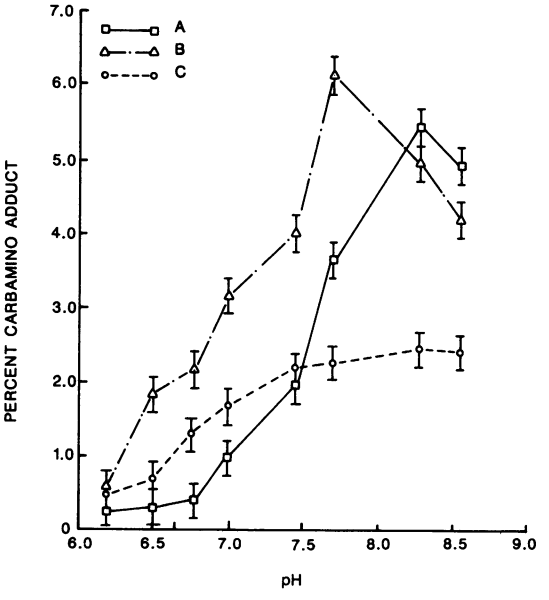

FIGURE 2 Graph of observed adduct fraction vs. pH for human zinc insulin. The amount of sodium (^{13}C) bicarbonate was maintained at 23 mM over the experiment.

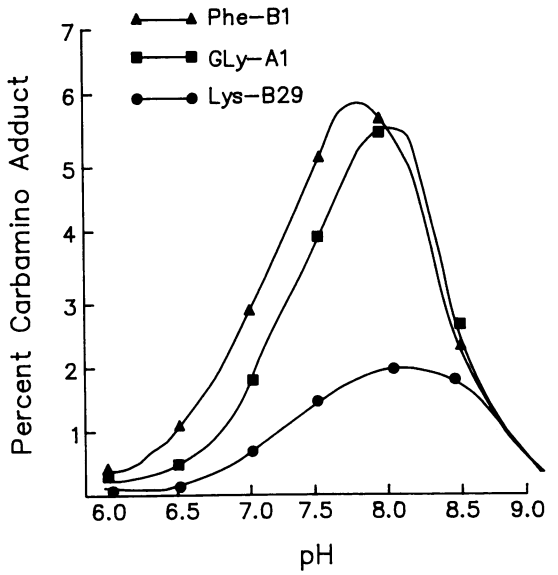

FIGURE 3 Graph of theoretical adduct fraction vs. pH of the available reactive amino groups on insulin at Gly-A1, Phe-B1, and Lys-B29 with pKa values of 7.2, 7.8, and 8.5, respectively.

linewidth may result from the T2 relaxation of the carbamino group, the 4-Hz linewidth sets the exchange rate at 12 s^{-1} for insulin and its adducts (9).

Zinc pork insulin also forms three carbamino adducts with (^{13}C) carbon dioxide at pH 7.4. The ^{13}C chemical shifts of the three adducts are nearly identical to those of the zinc human insulin, with signals at 165.2, 163.5, and 159.3 ppm. The intensity of the peak at 163.5 ppm is greatest at pH 7.4, similar to the observations for the human insulin. Peak C occurs as a single resonance at all pH values, consistent with the absence of the Tris adducts in this insulin sample prepared with no buffer.

Two carbamino adducts are observed for a solution of sodium pork insulin with (^{13}C) carbon dioxide at pH 8.1. These adducts resonate at 165.1 and 163.3 ppm. Little intensity is observed at 159.3 ppm where the signal from the third adduct is located in spectra of the Zn insulin. The intensities of the resonances for the adducts with the terminal amino groups are nearly equal. Addition of 0.5 mM Zn $(OAc)_2$ to the solution of sodium insulin and bicarbonate shifts the positions of the signals to 165.3 and 163.6, identical to the chemical shifts of the adducts in native Zn insulin. However, no signal is detected from the carbamino adduct corresponding to peak C. This resonance may be obscured by the larger signal from bicarbonate.

Circular Dichroism

The NMR method utilizes clinical concentrations of insulin at 0.75 mM, where the predominant form of material is expected to be the two zinc hexamer (10). The NMR label is located on the two terminal amino groups and the B29 lysine, and provides no information on the remainder of the protein. We have investigated the structure of the insulin in the presence of bicarbonate using circular dichroism. CD studies require a much lower concentration of protein (3 μM), where the predominant form of the insulin is expected to be the monomer in the equilibrium with the dimer (11). The ratio of the CD spectrum at wavelengths of 208 and 222 nm reflects the aggregation state of the insulin (10). Other changes at 208 nm may reflect changes in the amounts of alpha helix and random coil regions in the insulin.

The ratio of the absorbance at 208 and 222 nm is 1.7 for the Zn insulin. Addition of 23 mM sodium bicarbonate to a solution containing 0.2 A_{280} OD units of insulin does not change the ratio, as listed in Table I. The ratio is 1.6 for sodium insulin in buffer, but changes to 1.9 after addition of 23 mM sodium bicarbonate. Addition of Zn to the solution of sodium insulin does not change the ratio from 1.6. However, the ratio for this sodium insulin plus zinc does change to 1.9 when bicarbonate is added.

Carbon-13 NMR Studies of Ureido Adducts

Potassium cyanate is known to react with the amino groups of insulin in an irreversible fashion (12). Addition of 23 mM potassium (^{13}C) cyanate to a solution of Zn insulin at pH 7.4 results in the appearance of two new resonances at 160.5 and 159.9 ppm in the ^{13}C NMR spectrum. The ratio of the intensities are four to one, but no information

TABLE I
RELATIVE INTENSITIES (degrees cm^2/dmol) AND RATIOS OF 208- AND 222-nM BANDS IN CIRCULAR DICHROISM SPECTRA FOR INSULIN IN PRESENCE AND ABSENCE OF SODIUM BICARBONATE AND ZINC ACETATE

Insulin sample	Source	208 nM	222 nM	208 nM/222 nM
Sodium insulin	Pork	−29 ± 2	−19 ± 2	1.6 ± 0.1
Sodium insulin + HCO_3	Pork	−52 ± 2	−28 ± 2	1.9 ± 0.1
Sodium insulin + Zn	Pork	−28 ± 2	−19 ± 1	1.6 ± 0.1
Sodium insulin + Zn + HCO_3	Pork	−52 ± 2	−28 ± 2	1.9 ± 0.1
Zinc insulin	Human	−27 ± 1	−16 ± 1	1.7 ± 0.1
Zinc insulin + HCO_3	Human	−40 ± 2	−24 ± 2	1.7 ± 0.1

Zinc acetate final concentration, 100 μM.

concerning the relative roles of the terminal amino groups and/or lysine in the ureido adduct peaks can be determined. The linewidths of these resonances range from 8.0 to 8.5 Hz when proton decoupling is employed. In contrast, the linewidths of the carbamino adducts are within 4.0–4.5 Hz. No nuclear Overhauser effect was noted when broadband proton decoupling was applied.

DISCUSSION

Insulin in the plasma is assumed to exist in the only biologically active form, as a monomer of mol wt 5,660. Increasing concentrations prepared in buffered solutions lead to dimer formation by hydrophobic bonding at one surface of the monomer (11). The availability of zinc ions permit the further formation of the 2-Zn-hexamer, a rhombohedric soluble molecular form of insulin (13). The clinically available U-100 insulin, with a concentration of 0.75 mM, at pH 7.4 with zinc at a 0.33–1.0 molar ratio with the protein, should essentially be completely in the hexamer form, with dimer and monomer present only in small amounts in equilibrium with the rhombohedron (11, 16). On the surface of the dimer and the monomer, the Gly-A1, Phe-B1, and the Lys-B29 are found in pockets, which are available for reactivity of their amino groups. There is evidence from studies of organic–aqueous interface events, that absorption phenomena of insulin solutions may be initiated at these amino group sites (1). These reactive sites may also be the molecular surface required for insulin fibril formation, a biologically inactive and insoluble form of insulin. In fact, the early studies of Waugh (17) proposed that a linear continuous repeating structure of dimers may be the basis of fibril formation.

The addition of physiological levels of CO_2 to insulin solutions has been reported to increase their physical stability apparently by reducing the tendency for self-association to fibril formation (4). The present studies examine the extent to which carbamino-adduct formation occurs in insulin solutions, and propose that the resulting change in surface reactivity of the monomer or dimer may account for the observed improved physical stability of U-100 insulin in the presence of CO_2.

It has been demonstrated that this reversible reaction between carbon dioxide and the free amino groups in a protein generates carbamino adduct (6, 7, 8).

$$R\ddot{N}H_2 + CO_2 \rightarrow RN^+H_2CO_2^- \rightarrow R\ddot{N}HCO_2^- + H^+.$$

The quantity of the carbamino adduct is a function of the concentration of free amine, and the concentration of carbon dioxide in solution (7). These quantities are diminished at low pH, where the concentration of carbon dioxide is high but the amount of free amine is reduced. Similarly, at high pH, the level of free amine is greatly increased, but most of the carbon dioxide is converted to the unreactive bicarbonate form. When ^{13}C-enriched bicarbonate is mixed with a protein, new resonances from carbamino adducts can be detected and quantified. Because the natural abundance of ^{13}C is only 1.1%, even low levels of carbamino adducts are observed without interference from other resonances generated by the protein.

The insulin molecule contains three amino groups which can react with carbon dioxide to form carbamino adducts. The terminal amino groups of the two chains, Phe-B1 and Gly-A1, both exist in equilibrium between their $-NH_2$ and $-NH_3^+$ forms between pH 6.3 and 8.6. The Lys-B29 residue has an amino group which could form a carbamino adduct, but the quantity should be lower due to its protected environment and more basic pH relative to the terminal amino groups (2).

Zinc insulin of either human or pork structure forms three carbamino adducts. The only difference between the pork insulin and the human insulin is at position B30, which is occupied by an Ala in the pork, and a Thr in the human type. Two of the adducts have ^{13}C chemical shifts downfield from bicarbonate. These shifts are similar to the shifts observed for resonances from carbamino adducts of terminal amino groups in other proteins and are assigned to the carbamino adducts of the Phe-B1 and Gly-A1 units of insulin (7, 8). The measured pKas for these two groups range from 6.8 to 7.3 for Phe-B1, and from 7.7 to 8.0 for Gly-A1 (2). The signal intensity for peaks A and B in the ^{13}C NMR spectra of the carbamino adducts can be fit to calculated curves when pKa values of 7.2 and pH 7.8, respectively, are used in the calculation. Peaks A and B are assigned to the carbamino adducts of Gly-A1 and Phe-B1, accordingly. The chemical shift of the third adduct has a lower resonant frequency than the signal from bicarbonate. No other carbamino adducts have been detected in this region. This carbamino resonance is attributed to Lys-B29 based on the high pKa of the reacting amino group, and the observed low reactivity of Lys-B29 in labeling experiments of insulin fragments. Differences in the relative reactivity of these terminal amino moieties have been observed previously, and these differences may be attributed to formation of carbamino adducts which then render the amino groups unreactive (2).

Lys-B29 may not form the carbamino adduct with insulin in the sodium form. This residue is believed to reside in a protected portion of the protein which is normally not exposed to exogenous factors (14). Addition of bicarbonate to the concentrated hexameric form of sodium insulin required for NMR studies may not change the molecular structure sufficiently to open the Lys to chemical reaction. However, in the less aggregated form of insulin used for CD studies, addition of bicarbonate produces a dramatic change in the spectrum. This change to a more random coil form of insulin may be caused by the exposure of the Lys to carbon dioxide, followed by formation of the carbamino adduct. The introduction of negative charge at this site may produce a poor electrostatic interaction at the A chain terminus, because Lys-B29 may be involved in a hydrogen bond or salt bridge to the carboxyl

of A4 (14). The resonance from Lys-B29 in sodium insulin may be shifted downfield by such a change in insulin conformation leading to its being obscured by the bicarbonate signal.

Two lines of evidence suggest that carbamino adduct formation may occur on monomer or dimer molecular forms of insulin. First, it has been demonstrated that an increase in the number of negative charges on the insulin molecule leads to a decrease in the molecular weight of the complexes in the pH range of 7 to 10 (13). Formation of carbamino adduct takes a neutral charge on the terminal amino groups and converts it to a negative charge. Several specific dimer–dimer contacts would be impacted by the carbamino adducts. The amino group of Phe-B1 normally binds to the carboxyl of Glu-A14 of a paired insulin monomer (14). Introduction of a negative charge would disrupt this contact and potentially alter the dimer contact. The amino group of Gly-A1 appears to form an intramolecular salt bridge with an adjacent monomer at the carboxyl of A4 or by a hydrogen bond with a hydroxyl of A19 (14). Formation of a carbamino adduct at this Gly-A1 site would disrupt these salt bridge interactions, leading to a decrease in the molecular weight of insulin complexes.

The second line of evidence relates to the comparison of the irreversible ureido adducts with the carbamino adducts of insulin amino groups (12). We have characterized the ureido adducts by reacting zinc insulin with potassium (^{13}C) cyanate. The ureido adducts differ from the carbamino adducts in that no change is made in the charge of the amino groups, which are predominately unprotonated at pH 7.4 (14). The linewidths of the ^{13}C resonances are proportional to the size of the molecular complex and the mobility of the local protein environment, in the absence of complicating relaxation mechanisms and exchange broadening (15). The lack of a nuclear Overhauser effect demonstrates that little dipolar coupling occurs in the ureido adduct, and that chemical shift anisotropy is the primary relaxation mechanism. The irreversible ureido adduct is not exchange broadened, as is the carbamino adduct, so that the twofold greater linewidth of the ureido signals relative to that of the carbamino adducts is significant. The increased linewidth of the ureido adducts as compared with the carbamino adducts suggests that the molecular structure of the insulin undergoing adduct formation in the presence of sodium bicarbonate is small relative to the insulin structure where the terminal groups are ureido units. We propose that adduct formation occurs with monomers and/or dimers, which are in equilibrium with tetramers and the dominant 2-Zn-hexamer of insulin.

CONCLUSIONS

Insulin reacts with carbon dioxide to form carbamino adducts at physiologic pH and concentration of bicarbonate. Formation of the adducts is an equilibrium process that does not occur below pH 6.2, and is bounded by the available concentration of carbon dioxide below pH 9. Resonances from the carbamino sites can be observed using ^{13}C NMR spectroscopy and sodium (^{13}C) bicarbonate. These signals can be assigned to specific amino acids based on the known pK of the amino protons and the pH profile of the intensity of each peak. At pH 7.4, the primary site of carbamino adduct formation is the Phe-B1. The presence of the adduct places additional negative charge at the termini of the insulin chains. This charge may alter the tendency for self association of monomer and dimers as a result of reduced chemical reactivity at the amino termini.

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