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Evidence for the Compact Conformation of Monomeric Glucagon. Hydrogen–Tritium Exchange Studies†

Patricia A. McBride-Warren‡ and Richard M. Epand*

ABSTRACT: Glucagon, a polypeptide hormone of 29 amino acids, was subjected to tritium–hydrogen exchange according to the two-column technique of Englander. The primary structure of this molecule does not contain any covalent cross-links such as peptide cyclization or disulfide bonds; thus any slowly exchanging hydrogens would indicate a folding of the peptide chain. The hydrogen exchange of glucagon is described by more than one first-order rate constant. The exchange of the slowest class of hydrogens exhibits specific acid and base catalysis and is composed of approximately 8 hydrogens. The number of hydrogens in this kinetic class was found to be nearly constant (±2 hydrogens) within the entire pH interval investigated, showing that no conformational change had occurred between pH 1.7 and 4.0. It is suggested that most of these slowly exchanging hydrogens result from i to $(i + 3)$ hydrogen bonds of tetrapeptide β bends.

The rates of isotope exchange of hydrogen atoms in proteins have been shown to be a reflection of the conformation of these macromolecules (Hvidt and Nielsen, 1966). Studies with model compounds have been geared toward the elucidation of the factors involved in the kinetics of the exchange process (Berger *et al.*, 1959; Klotz and Frank, 1962, 1965; Nielsen, 1960; Leichtling and Klotz, 1966; Scarpa *et al.*, 1967; Kakuda *et al.*, 1971; Englander and Poulsen, 1969). With the

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‡ A Postdoctoral Fellow of R. M. E.

kinetics parameters of these model compounds at hand, it has been possible to examine the exchange process of more complicated systems. The hydrogen exchange of trypsin (Lenz and Bryan, 1969), lysozyme (McBride-Warren and Mueller, 1972), myosin (Segal and Harrington, 1967), ribonuclease (Woodward and Rosenberg, 1970), gramicidin S-A (Laiken *et al.*, 1969), and bacitracin A (Galardy *et al.*, 1971) has been interpreted in light of the information gained from earlier work.

Glucagon is a polypeptide hormone containing 29 amino acids, whose primary structure is well documented (Bromer *et al.*, 1956, 1971). We have measured the hydrogen exchange of this small biopolymer whose structure is not restrained by such factors as cyclization or disulfide bonds. It has been estimated that this protein contains 10–15% α helix in dilute aqueous solution (<1 mg/ml) (Blanchard and King, 1966; Gratzer *et al.*, 1968; Srere and Brooks, 1969) but aggregates to structures of higher helical content in more concentrated solutions. In addition, this peptide may aggregate to form gels from concentrated solutions over a period of several hours. This aggregation product has been shown to be composed of antiparallel β conformation (Beaven *et al.*, 1969; Epand, 1971). The aggregation of glucagon to form β structures is enhanced by increased concentrations of glucagon (Beaven *et al.*, 1969). It has been suggested on the basis of viscosity studies (Epand, 1971) and from physical measurements on the cyanogen bromide peptide of glucagon (Epand, 1972) that these peptides exist as compact spheres in dilute aqueous solution. It may thus be anticipated to contain slowly exchanging hydrogens and demonstrate distinct classes of hydrogen-exchange rates. The method of tritium–hydrogen exchange was used as a method for obtaining additional information about the structure of monomeric glucagon because of its high sensitivity at low peptide concentrations.

Experimental Section

Materials. Crystalline glucagon (lot 80C2900) was purchased from Sigma Chemical Co. and stored at 5°. Tritiated water (lot 559-298) was obtained from New England Nuclear Corp. at a specific activity of 5 Ci/g and was used at dilutions required to effect concentrations of up to 20 mCi/ml depending on the protein concentration being used. Guanidine hydrochloride (Schwarz–Mann) was the Ultra Pure grade; G-15 Sephadex was supplied by A. B. Pharmacia; acetonitrile (Matheson Coleman & Bell) was spectroquality grade.

Methods. Hydrogen–tritium exchange of glucagon was performed according to the gel filtration technique of Englander (1963). Glucagon at a concentration of 10.0 mg/ml was dissolved in tritiated buffer approximately 10 mCi/ml and 7 M in guanidine hydrochloride. This solution was allowed to stand at room temperature for 1 hr to effect the complete exchange-in of tritium. Guanidine hydrochloride was used as a denaturant in order to effect as rapid a tritiation of glucagon as possible. Exposure to tritium and guanidine hydrochloride for longer periods of time did not produce any increase in tritium incorporation. After about 1-hr tritiation at room temperature the solution was removed to the cold room at which time it was diluted fivefold with buffer maintained at 0°. This step simultaneously initiated the refolding of the glucagon to its native conformation by dilution of the guanidine hydrochloride as well as diluting out the solvent tritium. The solution was then administered immediately to a 2.5 × 33 cm column of Sephadex G-15, kept in the cold room at 4°, and an aliquot of this sample was saved for determination of tritium activity. The peptide was eluted in the void volume with 0.025 M citrate buffer at the working pH. All buffers contained 7% (v/v) acetonitrile to aid in the inhibition of the formation of gels (R. M. Epand, unpublished results). Dioxane has also been shown to have this effect (Beaven *et al.*, 1969). The effluent fractions were monitored by an ISCO Model UA-2 ultraviolet analyzer. The elution volume of solvent tritium was found to be almost exactly twice that of the protein-bound tritium and there was no overlapping of the two peaks. The protein peak was pooled and kept in an ice bath at 0°. An aliquot of the "pool" was removed for protein determination and tritium activity. Usually, the concentration of the protein solution collected from the long column was found to be around 0.4 mg/ml. If the concentration was greater than this value, the "pool" was diluted to effect a protein concentration of this value. At various times, aliquots of the pool were removed and passed through a 1.5 × 17.5 cm column of Sephadex G-15 to remove tritiated glucagon from solvent tritium which had exchanged out after passage through the long column.

In order to determine the point at which the exchange of tritium became unidirectional the correction factor from Laiken *et al.* (1969) was applied to all time calculations. Here, tritium exchange becomes unidirectional approximately one-sixth of the distance down the long column where one order of magnitude of the original tritium in the bulk solvent is removed.

Glucagon concentrations were measured spectrophotometrically using $E_{280}^{1\ mg/ml}$ of 2.30 (Kay and Marsh, 1959). Tritium activity in counts per minute per milliliter was measured in a Beckman DPM-100 liquid scintillation system. The scintillation fluid used was that recommended by the manufacturer and contained 5 g of 2,5-diphenyloxazole and 100 g of naphthalene per l. of dioxane.

The hydrogen–tritium exchange of racemized glucagon was also performed but only at pH 3.0. Glucagon was subjected to 1 N NaOH treatment for 12 hr at 25°, separated from solvent on a G-15 Sephadex column and lyophilized (Epand and Epand, 1972). The H–T exchange of the racemized protein was carried out exactly as in native glucagon.

Data Analysis. The exchange process was followed by calculating the number of hydrogens unexchanged per molecule of glucagon. The radioactivity per proton was calculated from the proton concentration of 7 M guanidine hydrochloride which we estimated from the data of Hade and Tanford (1967) to be 98 M. The values were also corrected for the tritium equilibrium isotope effect (Englander and Poulsen, 1969). The number of hydrogens remaining unexchanged at any time t can be written as a sum of first-order rate processes (Hvidt and Nielsen, 1960). Only the class of "slowly exchanging" hydrogens of glucagon was subjected to the rigorous kinetic analysis described below. The rate constant for this class of exchanging hydrogens was obtained by subjecting the data from this region to a least-squares analysis of the best straight line. A program designed for the Wang calculator yielded the slope of this line, which is the rate constant for this class of exchanging hydrogens, and the intercept which is the number of hydrogens exchanging in the class described by the rate constant obtained.

Results

Concentration Dependence. Table I gives the results of the exchange analysis at various concentrations of glucagon at pH 3.0. The rate constants given represent those describing

TABLE I: First-Order Rate Constants for Various Glucagon Concentrations at pH 3.0 and 0°.

Glucagon (mg/ml)	k (min^{-1})	Hydrogens/ Molecule
0.146	1.06×10^{-3}	6.3
0.174	1.15×10^{-3}	6.7
0.201	1.13×10^{-3}	7.1
0.355	1.14×10^{-3}	7.1
0.366	1.14×10^{-3}	7.1
0.741	4.74×10^{-3}	16.6
0.787	5.28×10^{-3}	18.4

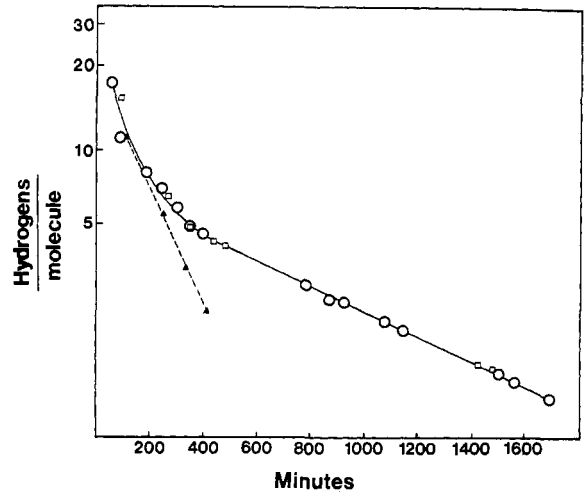

FIGURE 1: Exchange-out of glucagon at pH 3.0 and 0°. (○, □) Two different sets of data for native glucagon; (▲) racemized protein.

only the class of the most slowly exchanging hydrogens. As can be seen, the rate constants obtained from concentrations of glucagon below 0.4 mg/ml are essentially constant around an average value of 1.12×10^{-3} min^{-1} but increase more than 4-fold when the concentration of glucagon is increased to more than 0.7 mg/ml. The number of hydrogens involved in the class described by this rate constant is also increased at this concentration range. This concentration dependence of the hydrogen exchange can best be explained by the known tendency of the molecule to associate both to structures of higher helical content (King, 1965; Blanchard and King, 1966; Gratzer *et al.*, 1968) and to β-structures (Beaven *et al.*, 1969). The lowered tendency of glucagon to aggregate in dilute solution, at temperatures far removed from 30° and at low salt concentrations (Beaven *et al.*, 1969), support the results from H–T exchange (Table I) that below 0.4 mg/ml there is no effect of peptide concentration on the exchange process. Thus the peptide exists as a monomer under these conditions.

pH Dependence. Figure 1 shows the exchange-out of glucagon at pH 3.0 and 0° expressed as hydrogens remaining per molecule of glucagon as a function of time. The curvature of this plot indicates that, as in other proteins, the exchange process cannot be described by a single first-order process but as shown by Linderstrom-Lang (1955) by a sum of pseudo-first-order terms. Extrapolation of the linear portion of the curve to zero time yielded 7 hydrogens. This value was closely approximated at every pH investigated. Table II gives the observed rate constants and numbers of hydrogens found (as described under Data Analysis) for this slow class at each pH.

The early time portion of the pH 3.0 data extrapolates to approximately 29 hydrogens. This is in good agreement with the total number of 28 amide hydrogens capable of slow exchange at this pH.

The hydrogen exchange rate of simple amides is subject to specific acid and base catalysis (Hvidt and Nielsen, 1966) and can be expressed by the equation

$$k = k_0 + k_H(H^+) + k_{OH}K_{H_2O}/(H^+) \quad (1)$$

where k_H and k_{OH} are the specific acid and base catalytic constants, k_0 is the uncatalyzed rate constant, and K_{H_2O} is the disassociation constant of the solvent and equals 1.139×10^{-15} at 0° (Robinson and Stokes, 1965). The data were fitted to eq 1 using a nonlinear, least-squares program (Kakuda *et al.*, 1971). The variation of the observed rate constant with pH was described with k_0 taken as zero.

The mechanics of using this program involved estimating the values of k_H and k_{OH} and using these values as a first approximation in the program. These two values were determined by obtaining the slopes from plots of respectively (H^+) and (OH^-) *vs.* the observed rate constants. Using the slopes of these two plots as a first approximation, the Fortran program first finds the best values of k_H and k_{OH} which accurately describe the experimental data, then uses these values to calculate the curve described by eq 1. Table II lists the rate constants computed from this program and compares them to the rate constants experimentally obtained. The equation was fit to a standard deviation of 5.19×10^{-4} min^{-1}. Attempts to define the experimental data with eq 1, where k_0 was not equal to zero, yielded a standard deviation of 5.14×10^{-4} and computed rate constants almost identical with those obtained with a k_0 equal zero term. Since the largest difference between the two sets of computed data was 0.1×10^{-3} min^{-1} in the region of minimum exchange and the standard deviation was five times this value, k_0, if it actually does exist, could not be detected. The lowering of the standard deviation from 5.19×10^{-4} to 5.14×10^{-4} must also be seen as insignificant and it was therefore concluded that the data were adequately fit where the rate for any spontaneous exchange was considered negligible in the exchange process.

TABLE II: Hydrogens per Molecule in the Slowest Exchanging Class of Glucagon and Experimental and Computed First-Order Rate Constants, k (min^{-1}).

pH	Hydrogens/ Molecule	k (min^{-1})$_{exptl}$	k (min^{-1})$_{computed}$
1.7	8.3	5.51×10^{-3}	5.76×10^{-3}
2.0	9.5	3.40×10^{-3}	2.95×10^{-3}
2.4	9.6	1.44×10^{-3}	1.33×10^{-3}
2.7	8.3	1.16×10^{-3}	0.96×10^{-3}
3.0	7.1	1.14×10^{-3}	1.05×10^{-3}
3.4	7.2	1.34×10^{-3}	2.04×10^{-3}
3.7	7.1	3.16×10^{-3}	3.89×10^{-3}
4.0	10.4	8.20×10^{-3}	7.68×10^{-3}

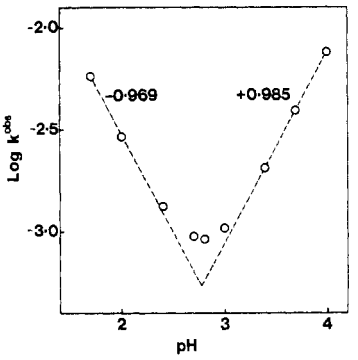

FIGURE 2: k_{obsd} *vs.* pH. The dashed line yields the estimation of the slopes obtained in the high hydrogen and hydroxyl ion concentration regions.

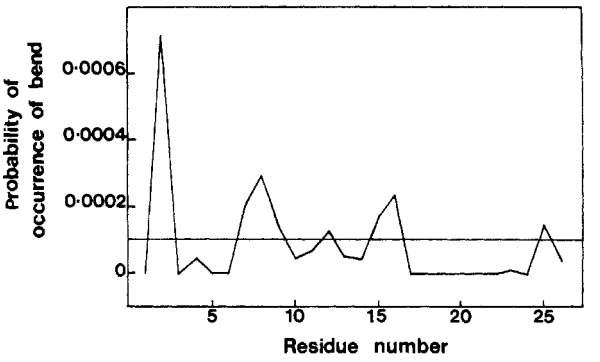

FIGURE 3: Folding probability of glucagon. Cutoff line at 10^{-4} probability arbitrarily chosen according to the method of Lewis *et al.* (1971).

Having obtained the values of the acid and base catalytic constants it was now possible to calculate the pH of minimum exchange by the equations of Leichtling and Klotz (1966)

$$(H^+)_{min}^2 = k_{OH}K_{H_2O}/k_H \quad (2)$$

$$k_{min} = k_0 + 2(k_H k_{OH} K_{H_2O})^{1/2} \quad (3)$$

Table III gives the values of pH_{min} and k_{min} calculated from values of k_H and k_{OH} found in the computational analysis of the data.

A plot of the log rate constants *vs.* pH should give a slope of -1 and $+1$ for pure acid and base catalysis, respectively. Figure 2 shows a slope of -0.969 for the acid-catalyzed portion of the exchange process and $+0.985$ for the base-catalyzed exchange.

Exchange of Racemized Glucagon. Figure 1 includes data from the exchange-out of glucagon which had been subjected to 1 N NaOH for 12 hr. The exchange was carried out at pH 3.0 and 0° and a rate constant of 5.20×10^{-3} min^{-1} was obtained. This was an increase of 4.56 times the value obtained for the eight slowly exchanging hydrogens under the same experimental conditions with native glucagon. An extrapolation of the data to zero time indicated 20 hydrogens to be within this kinetic class. Since there are a possible total of 28 exchangeable amide hydrogens at pH 3.0, this leaves 8 hydrogens unaccounted for which probably have very fast exchange rates.

Discussion

Hydrogen–tritium exchange has demonstrated that glucagon contains approximately eight protons which exchange with a rate constant of 0.937×10^{-3} min^{-1} at the pH_{min} of 2.79. The rate of this process is about one order of magnitude slower than that found for simple amides (Berger *et al.*, 1959; Nielsen, 1960; Klotz and Frank, 1962, 1965; Feidelseit, 1967) or for randomly coiled polypeptides such as poly-DL-alanine (Englander and Poulsen, 1969) or oxidized ribonuclease (Woodward and Rosenberg, 1970). This class of slowly exchanging hydrogens disappears when glucagon is partially racemized by treatment with 1 N NaOH (Figure 1), demonstrating the dependence of these hydrogens on the stereochemistry of the molecule. The exchange rates of peptides, polypeptides, and proteins have been recently shown to depend only on nearest neighbor effects (Molday *et al.*, 1972). The existence of a class of hydrogens in glucagon with an exchange rate much slower than those of model peptides clearly demonstrates the existence of structured regions of this peptide.

TABLE III: Kinetic Parameters of H–T Exchange of the Glucagon Monomer at 0°.

pH_{min}	k_{min} (min^{-1})	k_H (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})
2.79	0.937×10^{-3}	0.287 ± 0.0228	(6.719 ± 0.396) 10^8

In comparison to other proteins recently studied the k_{min} of the slowest class of exchanging hydrogens in glucagon appears to fall within a predictable range. A rate constant of 2.5×10^{-4} min^{-1} describing the slowest class of 4 exchanging hydrogens of gramicidin S-A was explained in terms of the intramolecular hydrogen bonding (Laiken *et al.*, 1969). The rate constant of 2.1×10^{-3} min^{-1} was also used as evidence of an intramolecular hydrogen bond in bacitracin A (Galardy *et al.*, 1971). The very low value obtained for the slowest class of exchanging hydrogens in lysozyme (5.1×10^{-6} min^{-1} when corrected to 0°) probably reflects exchange of helical and β structures located in the interior of the protein where very little contact with solvent occurs (McBride-Warren, 1971a,b). The magnitude of the slow rate constant from lysozyme, compared to those from gramicidin S-A, bacitracin A, and glucagon, must be seen as a reflection of the relative size difference of the molecules.

Class Size. The number of hydrogens falling into the class with the slowest exchange rate is relatively independent of pH and is equal to 8.5 ± 2.0 (Table II). This suggests that the glucagon monomer has a specific, nonrandom structure which is insensitive to pH in the region studied. It also shows that a large fraction of the exchangeable hydrogens fall within the slowest class of exchanging hydrogens. The small size of the molecule would seem to preclude inaccessibility to solvent for such a large number of hydrogens and therefore necessitated the assumption that these slowly exchanging hydrogens are hydrogen bonded.

Lewis *et al.* (1971) recently proposed a mechanism for the folding of polypeptide chains of proteins based on known crystallographic structures and found that it was possible to predict with a reliability of about 80% the position of β bends

in the native conformation of proteins. Glucagon was analyzed for the probability of occurrence of such bends according to the procedure outlined by these investigators. Figure 3 shows that by arbitrarily assuming a cutoff probability of 10^{-4}, nine tetrapeptide bends were predicted for this polymer. This approximate theory gives surprisingly good agreement with the number of slowly exchanging hydrogens found by tritium exchange.

In summary, the hydrogen exchange of native glucagon confirms previous indications that glucagon exists in a compact conformation in solution (Epand, 1971, 1972). The relatively large number of hydrogens involved in the slowest class of exchanging hydrogens strongly supports this idea and the exchange of racemized glucagon demonstrates the stereochemical dependence of the slowly exchanging class of hydrogens.

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