Specific Tritium Labeling of γ-Carboxyglutamic Acid in Proteins†

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ABSTRACT: A specific method has been devised for the incorporation of tritium (T) into γ-carboxyglutamic acid (Gla) residues in proteins. This is achieved by equilibration of the protein with tritiated 0.05 M deuterium chloride (DCl) in D_2O. After removal of the equilibration solvent, the dry protein is heated at 110 °C in vacuo for 3–6 h. The resulting thermal decarboxylation of Gla residues promotes tritium incorporation at the γ carbon of newly formed glutamic acid residues. More than 99% of the incorporated tritium is in glutamic acid residues after acid hydrolysis. The efficiency of this *exchange decarboxylation* reaction is 0.95 ± 0.14 mol of T incorporated per mol of Gla decarboxylated, compared to the predicted value of 2.0. Labeling efficiency is only slightly reduced to 0.69 mol of T per mol of Gla decarboxylated when the protein is equilibrated with tritiated 0.05 M HCl. Tritium-labeled proteins and peptides produced by this method are normal in every respect except for the substitution of γ-methylene-tritiated glutamic acid for Gla residues. Thus, the method offers great promise for (1) isolation, purification, and sequencing of Gla-containing proteins and peptides, (2) preparation of tritium-labeled substrates for studying vitamin K dependent carboxylation by tritium release, and (3) selective autoradiographic localization of Gla-proteins in tissues without prior purification.

γ-Carboxyglutamic acid (Gla)[1] is an important constituent of a rapidly growing list of proteins. Gla residues are implicated in the (1) binding of Ca^{2+} ions (Nelsestuen & Suttie, 1973; Stenflo & Ganrot, 1973; Märki et al., 1977; Sperling et al., 1978), (2) adsorption of proteins to insoluble salts of Ca^{2+} (Hauschka et al., 1975; Hauschka & Gallop, 1977; Poser & Price, 1979) and Ba^{2+} (Skotland et al., 1974), (3) interaction of proteins with phospholipid micelles and membranes (Gitel et al., 1973; Nelsestuen et al., 1976; Nelsestuen & Lim, 1977), and (4) Ca^{2+}-mediated proteolytic activation of proenzymes (Esmon et al., 1975a; Bajaj et al., 1975). Biosynthesis of Gla occurs posttranslationally and involves vitamin K dependent carboxylation of specific glutamic acid residues in the polypeptide chain (Esmon et al., 1975b; Friedman & Shia, 1976; Olson & Suttie, 1977). Gla residues are found in abundance in the NH_2-terminal regions of prothrombin (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974, 1975) and other vitamin K dependent plasma proteins including factors VII, IX, and X, protein C, protein S, and protein Z [reviewed by Stenflo (1977)].

Mineralized osseous tissue contains abundant quantities of a small Gla-rich protein named osteocalcin (Hauschka et al., 1975, 1978; Price et al., 1976). Gla-proteins also occur in sites of ectopic calcification such as renal stones (Lian et al., 1977) and atherosclerotic plaque (Lian et al., 1976) and in urine (Fernlund, 1976). Other tissues involved in the transport of Ca^{2+} exhibit Gla-protein synthesis, including kidney (Hauschka et al., 1976; Traverso et al., 1979), placenta (Friedman et al., 1979a), and chick chorioallantoic membrane (Tuan, 1979). Recently, vitamin K dependent Gla-protein biosynthesis has been demonstrated in lung and spleen (R. Bell, personal communication) and pancreas (H. P. Traverso, personal communication).

Specific procedures for labeling Gla have long been sought in this and other laboratories. Traditionally, the methods employed for studying Gla have been (1) alkaline hydrolysis and amino acid analysis (Hauschka, 1977), (2) mass spectrometry (Stenflo et al., 1974; Magnusson et al., 1974; Thøgersen et al., 1978), (3) in vitro microsomal carboxylation in the presence of vitamin K and $^{14}CO_2$, yielding [γ-*carboxyl*-^{14}C]Gla (Esmon et al., 1975b; Hauschka et al., 1976), and (4) tritiated diborane reduction followed by isolation of the Gla derivative 5,5′-dihydroxyleucine (Zytkovicz & Nelsestuen, 1975). The latter procedure unfortunately requires acid hydrolysis and ion-exchange chromatography to resolve the labeled Gla derivative from derivatives of aspartic acid (homoserine) and glutamic acid (hydroxynorvaline) which are also heavily labeled (Zytkovicz & Nelsestuen, 1976). In vitro labeling with $^{14}CO_2$ is relatively specific for Gla, but the efficiency of incorporation is very low and one often does not have access to a suitably defined microsomal system to yield significant amounts of labeled Gla-protein.

An obvious focal point for labeling is the γ-carboxyl group of Gla. When Gla is decarboxylated in acid (Hauschka et al., 1975), the γ-carboxyl group is replaced by a hydrogen atom. Free Gla heated in tritiated 6 M HCl is readily converted to tritiated glutamic acid (unpublished experiments). The problem is how to achieve the same reaction in proteins and peptides without extensive hydrolytic cleavage. Attempts to achieve Gla decarboxylation in dry trifluoroacetic acid (Cl_3AcOH) (R. Hiskey, personal communication) or in Cl_3AcOH anhydride were only partially successful. About half of the Gla in osteocalcin decarboxylates in Cl_3AcOH, but when the reaction is done in tritiated Cl_3AcOH, label is incorporated into many amino acids in a nonspecific fashion (unpublished experiments).

Thermal decarboxylation of Gla-peptides was first observed by Stenflo et al. (1974) and Magnusson et al. (1975), who found that dry heating at 150 °C for 10–30 min caused complete conversion of Gla residues (in the acid form) to glutamic acid. Magnusson et al. (1975) cleverly exploited this property for identification of Gla-peptides by "thermal diagonal" electrophoresis. Poser & Price (1979) have recently

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[1] Abreviations used: Gla, γ-carboxyglutamic acid; Gla-protein, any protein containing Gla residues in peptide linkage; T, tritium; D, deuterium; TDCl, pure T_2O diluted in 99.7% D_2O and DCl to the concentration indicated.

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extended this method to the Gla-protein from bovine bone (bovine osteocalcin). By restriction of water from the reaction during 3 h at 110 °C in vacuo, the protein could be decarboxylated, apparently without introducing other changes.

We reasoned that thermal decarboxylation should be a feasible method for introducing tritium or deuterium into Gla-proteins if exchangeable hydrogens in the protein were first replaced by the desired isotope. While the source of the hydrogen atom which fills the vacancy on the γ-carbon atom resulting from decarboxylation is unknown, it must derive from some exchangeable position in the dry protein. This paper reports the successful development of this reaction into a highly specific method for labeling Gla-proteins.

Materials and Methods

Isotopes. Tritiated water made up in 99.7% pure deuterated water (T_2O in D_2O) was provided at a specific activity of 1.42 Ci/mL by New England Nuclear (Boston, MA). D_2O was obtained from the same source, and concentrated 38% DCl was purchased from ICN (Irvine, CA). Stock equilibration solutions of T_2O in 0.05 M DCl were prepared by volumetric dilution to final specific activities ranging from 85 to 426 mCi/mL or 1700 to 8520 dpm/nmol of D. These solutions are referred to as "0.05 M TDCl", and at this specific activity the fraction of hydrogen atoms which are actually T ranges from 2.6×10^{-5} to 1.3×10^{-4}. The background of nonvolatile radioactivity in the 0.05 M TDCl was less than 10^{-10} of the total disintegrations per minute.

Proteins Containing Gla Residues. Osteocalcin, the Gla-protein of bone, was purified from chicken bone by EDTA extraction, gel filtration, and DEAE-Sephadex column chromatography as previously described (Hauschka et al., 1975; Hauschka & Gallop, 1977). This protein has a molecular weight of 6500 and contains ~57 amino acid residues of which 4 are Gla and 7 are glutamic acid. The protein concentration was determined both by amino acid analysis of acid hydrolysates and by absorbance at 276 nm, where $\epsilon = 3800\ M^{-1}\ cm^{-1}$ (Hauschka & Gallop, 1977). Purified human prothrombin was generously provided by Dr. R. Rosenberg (Children's Hospital, Boston, MA). The purified F1 fragment of human prothrombin was a gift of Dr. K. Koehler (University of North Carolina, Chapel Hill, NC). Amino acid compositions of prothrombin (582 residues) and its Gla-rich F1 fragment (156 residues) were found to agree closely with values published by Magnusson et al. (1975). Partial decarboxylation of osteocalcin, prothrombin, and F1 fragment was achieved by heating for 3 h at 110 °C in vacuo after drying from 0.05 M HCl (Poser & Price, 1979). This treatment caused the following reduction in Gla content: 3.83 → 0.90 residue of Gla per molecule of osteocalcin; 10.42 → 2.25 residues of Gla per molecule of prothrombin; and 9.87 → 0.87 residue of Gla per molecule of F1 fragment. These partially decarboxylated proteins were used as controls to study the specificity of the tritiation reaction.

Proteins Devoid of Gla Residues. Thermolysin was kindly provided by Drs. B. Holmquist and B. Vallee (Harvard Medical School, Boston, MA). Statherin, the tyrosine-rich acidic peptide of saliva, was furnished by Dr. D. I. Hay (Forsyth Dental Infirmary, Boston, MA). Carbonic anhydrase from bovine erythrocytes, bovine trypsin, and whale skeletal myoglobin were obtained from Sigma Chemical Co. (St. Louis, MO) at the highest available purity. Previous studies (Hauschka, 1977) had shown these proteins to be devoid of Gla residues.

Amino Acid Analysis. Quantitation of Gla and other amino acids in both alkaline and acid hydrolysates was achieved by established methods (Hauschka, 1977). Tritium profiles of protein hydrolysates separated on a Beckman 121M amino acid analyzer were measured both by flow scintillation counting (Hauschka et al., 1976) and by direct collection of the effluent stream after the ninhydrin reaction bath and colorimeter. In the latter case, 10 mL of scintillation fluid was added to each 200-μL (1-min) fraction. Tritium was counted with an efficiency of 34.6% by this procedure.

Liquid Scintillation Counting. Aqueous samples (0.02–1.0 mL) were dispersed in 10 mL of scintillation fluid (Formula 963; New England Nuclear) and counted at 0 °C in the tritium window of an Intertechnique liquid scintillation spectrometer. Efficiency was calibrated by internal standards of tritiated water (New England Nuclear) and was 40.4% for tritium unless otherwise noted. Stock solutions of 0.05 M TDCl were diluted to 2×10^{-5} in 0.05 M HCl before counting for determination of specific activity.

High-Performance Liquid Chromatography (LC). Osteocalcin was separated from other substances on a 4.4 × 300 mm μBondapak-CN column using a Waters Associates (Milford, MA) liquid chromatograph operated at 1200 psig, 23 °C, and 2.0 mL/min. The buffer was 0.01 M triethylamine–formate, pH 4.40 (TEAF buffer). This provided the same degree of resolution and recovery of applied protein (>90%) as the triethylamine–phosphate buffer system described by Rivier (1978) and had the added advantage of being volatile. Triethylamine was obtained from Pierce Chemical Co. (Rockford, IL). A linear gradient of acetonitrile (Burdick and Jackson, Muskegon, MI) in TEAF buffer was used to elute the osteocalcin in a sharp band. Protein in the effluent was monitored by absorbance at 210 nm. Radioactivity in 1-mL fractions of the effluent stream was counted by liquid scintillation at an efficiency for tritium of 34.6%.

Vacuum System. A Virtis lyophilizer, operating at a pressure of 0.1 mmHg, was utilized for lyophilization and evacuation of samples prior to sealing for decarboxylation. Access of tritium to the main system was largely prevented by a series of liquid nitrogen and P_2O_5 traps.

Procedure for Tritium Incorporation at Gla Residues. Proteins were dissolved in cold 0.05 M HCl and dialyzed against this same solvent in Spectrapor 3 membrane tubing (Spectrum Industries, Los Angeles, CA) to remove metal ions. Aliquots of the clear solutions containing 0.05–1.6 mg of protein were dispensed into acid-washed 18 × 150 mm Pyrex tubes, with other samples being set aside for amino acid analysis and determination of protein concentration. The Pyrex tubes were frozen in liquid nitrogen and then lyophilized. One milliliter of 0.05 M DCl in D_2O was added to each tube of protein, and after 30 min at 20 °C the clear solutions were again lyophilized, yielding the dry, largely deuterated protein (Hvidt & Linderstrøm-Lang, 1955). Eighty microliters of 0.05 M TDCl was then allowed to wet the dried protein film for 10 min at 20 °C. The sample was then frozen at −196 °C and lyophilized to dryness. Tubes containing dry, tritium-exchanged protein were pulled to a constriction with a torch and then replaced on the vacuum system. Evacuation to below 0.1 mmHg was achieved within 2 min and was aided by warming the tube to 60 °C (Hvidt & Linderstrøm-Lang, 1955) before sealing. For achievement of the highest efficiency of Gla labeling, loss of protein-bound tritium by back-exchange with H_2O (and trap ice) must be prevented. Therefore, a small pellet (~500 μL) of 0.05 M TDCl or THCl was maintained in proximity to the exchanged, dry protein (using a 3-cm side arm blown in the sample tube) during final evacuation and sealing.

Table I: Procedure for Tritium Incorporation into γ-Carboxyglutamic Acid Residues by Decarboxylation

step	total tritium in sample (dpm)	mol of tritium bound per mol of protein
800 μg of osteocalcin (123 nmol) in Pyrex tube	0	0
1. dissolved in 0.05 M DCl, lyophilized	0	0
2. dissolved in 80 μL of 0.05 M TDCl[a]	1.51×10^{10}	7.20×10^{4}
3. lyophilized to remove TDCl	6.64×10^{6}	31.6[b]
4. sealed in vacuo, heated 3 h at 110 °C[c]		
5. dissolved in 1 mL of 0.05 M HCl, lyophilized	2.42×10^{5}	1.15
6. repeat step 5	2.10×10^{5}	1.00
7. repeat step 5	1.79×10^{5}	0.853
8. dissolved in 1 mL of 6 M HCl, hydrolyzed 18 h at 110 °C, flash evaporated	7.91×10^{4}	0.444[d]

[a] Specific activity = 1706 dpm/nmol of D. [b] This is less than the total exchangeable hydrogens in osteocalcin (51 side chain + 49 peptide = 100) and reveals that some loss of tritium by back-exchange with atmospheric water vapor has occurred before sealing in vacuo. [c] 2.89 residues of Gla decarboxylated to glutamic acid per molecule of protein in step 4. [d] 0.444/2.89 = 0.154 mol of tritium incorporated per mol of Gla decarboxylated.

Proteins were heated in these sealed tubes at 110 °C for various times and then cooled, opened, and transferred to clean tubes with 3 mL of 0.05 M HCl. This solvent was removed by lyophilization. Aliquots were removed at this step for alkaline hydrolysis and determination of the number of Gla residues which had been decarboxylated during the heating at 110 °C. The lyophilization process was then repeated up to four more times with 1 mL of 0.05 M HCl in each step (see Table I). Although Gla-proteins could be clearly discerned at this point by their higher levels of nonvolatile radioactivity, it was necessary to hydrolyze them in 6 M HCl (110 °C, 18 h) and flash evaporate the acid twice at 50 °C (1 cmHg pressure) in order to reduce the background to tolerable levels. The dry hydrolysate was dissolved in 1 mL of 0.05 M HCl, and aliquots were analyzed for tritium, amino acid concentration and composition, and glutamic acid labeling by methods described above.

Exchange of solvent hydrogen with hydrogen atoms bound to the γ carbon of glutamic acid is known to occur in strong acid at high temperature (Rattner et al., 1940; Manning, 1970). Obviously, loss of T from this position during acid hydrolysis would jeopardize the calculated incorporation of isotope in these experiments. Thus, rates of T exchange at the γ carbon were measured. Rattner et al. (1940) had observed for glutamic acid in ~6 M DCl at 100 °C that D is incorporated at the γ carbon with a $t_{1/2}$ of ~96 h. Tritium exchanges into this position with a $t_{1/2}$ of ~22 h in 6 M DCl at 100 °C (P. V. Hauschka, unpublished experiments). Exchange *out* of T incorporated at the γ carbon is much slower, however. Tritiated [γ,γ-$^3H_2$]glutamic acid was prepared by heating pure synthetic Gla in tritiated 6 M DCl at 100 °C for 6 h (Hauschka, 1977) and then flash evaporating to dryness 5 times (2.05 atoms of T incorporated per molecule of glutamic acid). Aliquots of [γ,γ-$^3H_2$]glutamic acid were heated in 6 M HCl or 6 M DCl for various periods at 100 °C and then flash evaporated to dryness 3 times. The observed $t_{1/2}$ values for exchange-out of T were 61 h in 6 M DCl and 108 h in 6 M HCl. The latter value was used for correction purposes; thus, the hydrolysis (6 M HCl, 100 °C, 24 h) of proteins labeled with T by exchange decarboxylation of Gla residues causes a loss of only 15% of the T from the γ carbon of glutamic acid residues.

Results

One objective of these studies was to demonstrate a direct relationship between the number of Gla residues lost by decarboxylation and the amount of tritium incorporated into the resulting glutamic acid residues. The reaction scheme for exchange and incorporation of tritium is shown in Figure 1. Thermal decarboxylation of Gla residues is of primary importance because it allows the γ-carbon atom to form a new bond to a hydrogen (or tritium as the case may be). The kinetics of the thermal decarboxylation reaction for Gla in proteins are shown in Figure 2. Osteocalcin from chicken bone exhibits two distinct classes of Gla residues in that about

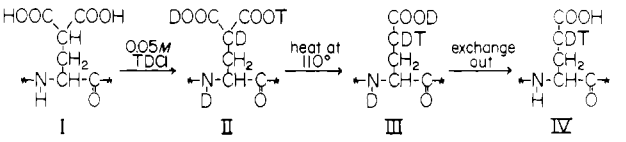

FIGURE 1: Reaction scheme for tritium (T) incorporation during decarboxylation of γ-carboxyglutamic acid (Gla). Proteins and peptides containing Gla residues (I) are equilibrated briefly with high specific activity tritiated water made up in 0.05 M DCl and then lyophilized. Approximately 10^{-4}–10^{-5} of the exchangeable hydrogens indicated by D are actually T, and these T atoms are presumably distributed randomly among all the exchangeable sites. The tritium-labeled deuterated material (II) is heated in vacuo at 110 °C for up to 6 h, during which time Gla decarboxylation occurs with transfer of tritium to the γ carbon of the newly formed glutamic acid residue (III). Back-exchange of other hydrogens (peptidyl, carboxyl, amino, and side chain) is achieved by dialysis or repeated lyophilization from 0.05 M HCl, yielding the specifically labeled product (IV). Hydrolysis of IV shows that all of the tritium label (>99%) is in glutamic acid. The maximum observed efficiency would be 2.00 mol of T incorporated per mol of Gla decarboxylated, depending on the extent of isotope equilibration with the relevant exchangeable sites.

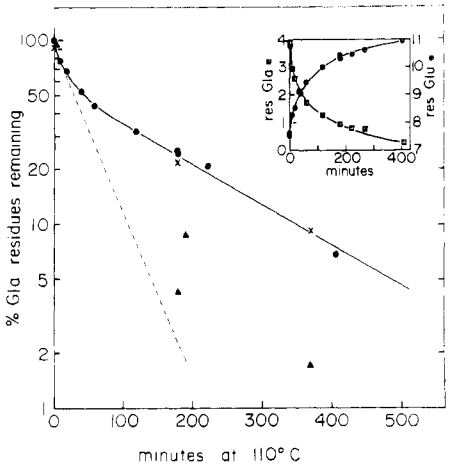

FIGURE 2: Kinetics of decarboxylation of Gla residues in proteins. Samples of protein lyophilized from 0.05 M TDCl were heated in vacuo for the indicated time. Alkaline and acid hydrolysates were used for determination of the amino acid composition. Chicken bone osteocalcin (●), human prothrombin (×), and the amino-terminal fragment 1 of human prothrombin (▲) were studied. Inset: residues of Gla (⊡) and glutamic acid (⊙) per molecule of osteocalcin; the sum of Gla + glutamic acid = 11.28 ± 0.04 residues for the 12 separate samples measured. The Gla decarboxylation kinetics for bovine osteocalcin reported by Poser & Price (1979) are shown for comparison (- - -).

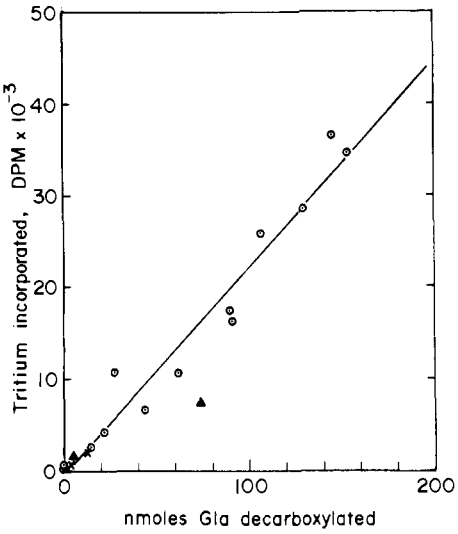

FIGURE 3: Proportionality of tritium incorporation and Gla decarboxylation. Osteocalcin (⊙), prothrombin (×), and prothrombin fragment 1 (▲) were studied. The linear least-squares fit to the data (—) shows a correlation coefficient of 0.968; the corrected slope is 0.155 mol of tritium incorporated per mol of Gla decarboxylated. The total nanomoles of Gla converted to glutamic acid during the in vacuo heating step at 110 °C (10–405 min) was measured by amino acid analysis. Incorporated tritium was measured after four lyophilizations from 0.05 M HCl, hydrolysis in 6 M HCl, and flash evaporation. Tritium specific activity was 1706 dpm/nmol of D.

two residues are converted to glutamic acid with $t_{1/2} \simeq 14$ min and the other two are decarboxylated much more slowly ($t_{1/2} \simeq 180$ min). Previously reported data for bovine osteocalcin (Poser & Price, 1979) showed a single class of three Gla being lost with $t_{1/2} = 32$ min (dashed line in Figure 2). Differences in the Ca^{2+} binding properties of chicken osteocalcin (Hauschka & Gallop, 1977) and the bovine protein (Price et al., 1977) are undoubtedly related to the protein environment surrounding each Gla residue, and this environment apparently also affects the rate of thermal decarboxylation. Human prothrombin loses its Gla residues with the same kinetics as chicken osteocalcin, while the F1 fragment is rather more sensitive to thermal decarboxylation (Figure 2).

Incorporation of tritium into Gla-proteins by thermal decarboxylation after tritium exchange is shown in Figure 3. There is a very low background for unheated or previously decarboxylated Gla-proteins, and the tritium disintegrations per minute incorporated is linearly related to the nanomoles of Gla lost by decarboxylation. The slope in Figure 3 gives a value of 0.155 mol of tritium incorporated per mol of Gla decarboxylated. In Table I the procedure for tritium incorporation is followed step by step. The net result for this single sample was 0.444 mol of T incorporated per mol of osteocalcin. Because the measured loss of Gla residues was 2.89 residues of Gla per molecule, one may calculate an efficiency of labeling of 0.154 mol of T per mol Gla decarboxylated. The reason why these numbers are considerably lower than the expected value of 2.00 is related to back-exchange of the tritiated protein before decarboxylation, as discussed below (see also Table III).

The specificity of the tritiation reaction was tested on various Gla-proteins and on proteins devoid of Gla (Table II). The level of tritium incorporation is obviously highest for the Gla-containing proteins osteocalcin, prothrombin, and prothrombin fragment 1. The background of incorporation in unheated control samples is very low; for osteocalcin it is only 0.7% of the 3-h heated sample. Partial decarboxylation reduces the absolute amount of tritium incorporation proportional to the fraction of Gla residues destroyed (Table II).

Table II: Specificity of Tritium Incorporation into Various Proteins[a]

protein	h at 110 °C	mol of T incorpd/ mol of Gla decx	nmol of T incorpd/ μmol of total Glu[b]	dpm in Glu/μmol of total Glu[b]
osteocalcin	0		0.25	
osteocalcin	3	0.160	42.33	72 240
osteocalcin (79% decx)[c]	0		0.32	
osteocalcin (79% decx)[c]	3	0.179	8.64	14 700
prothrombin	3	0.116	13.46	22 940
prothrombin (78% decx)[c]	3	0.206	3.78	6 450
prothrombin F1	0		0.36	
prothrombin F1	3	0.068	27.95	47 650
prothrombin F1 (91% decx)[c]	0		0.47	
prothrombin F1 (91% decx)[c]	3	0.206	6.28	10 720
thermolysin	0		0.15[d]	<30
thermolysin	3		2.00[d]	<170
trypsin	0		0.63[d]	<100
trypsin	3		2.40[d]	<200
statherin	0		0.37[d]	
statherin	3		0.46[d]	
carbonic anhydrase	3		1.31[d]	
myoglobin	3		1.22[d]	

[a] Samples were carried through all eight steps of the standard method shown in Table I. [b] Total micromoles of glutamic acid in the acid-hydrolyzed labeled protein. [c] % decx: partially decarboxylated by 3 h at 110 °C in vacuo prior to equilibration with 0.05 M TDCl; the percent of the total Gla residues converted to glutamic acid by this prior heating is indicated. [d] Less than 5% of this radioactivity is in glutamic acid by radiochromatography.

For the control proteins which are devoid of Gla, there is a very low level of tritium incorporation (Table II). Chromatography of the acid hydrolysates of trypsin and thermolysin was undertaken to identify the small amount of nonvolatile radioactivity in these samples. There was no detectable tritium associated with glutamic acid in these samples, with only traces of radioactivity eluting in the region of glycine and alanine. Thus, in the third column of Table II, computation of the tritiated glutamic acid produced in each protein by the standard labeling procedure shows the extremely high specificity of this method for labeling Gla-proteins.

Table III shows the results of two exchange techniques and various isotopic ratios in the equilibrating solvent on the efficiency of tritium incorporation. In expt 2 (Table III) the rapid back-exchange of protein-bound T with atmospheric water vapor has been prevented, and the efficiency of labeling is 0.95 ± 0.14 mol of T per mol of Gla decarboxylated, about half of the theoretical value of 2.00. In a similar experiment with 0.05 M THCl instead of TDCl, the efficiency is reduced slightly to 0.69 ± 0.09 mol of T incorporated per mol of Gla decarboxylated. Thus, the primary (kinetic) isotope effect $(k_T/k_D)/(k_T/k_H)$ is only about 1.4. Table III also shows that when the atmospheric back-exchange is allowed to proceed for 2 h (expt 1), the efficiency of T incorporation is reduced about sixfold. There is still a small isotope effect of ~1.2 in expt 1 (Table III).

Treatment of Gla-proteins by the standard incubation procedure results in highly specific incorporation of tritium into glutamic acid. Figure 4 shows that for an acid hydrolysate of tritiated, decarboxylated osteocalcin more than 99% of the radioactivity elutes at the position of glutamic acid on the

Table III: Isotope Effect on Tritium Incorporation into Osteocalcin[a]

isotopic composition of exchange solvent	h at 110 °C	total incorpn (dpm/nmol of protein)	mol of T incorpd/ mol of Gla decx
expt 1			
99.7% D/0.3% H	0	14	
99.7% D/0.3% H	3	1009	0.144
50% D/50% H	0	8	
50% D/50% H	3	1024	0.146
10% D/90% H	0	22	
10% D/90% H	3	831	0.118
expt 2			
99.7% D/0.3% H	0	12 ± 7	
99.7% D/0.3% H	6	3152 ± 116	0.95 ± 0.14
6% D/94% H	6	2446 ± 73	0.69 ± 0.09

[a] Each tube contained 123 nmol of osteocalcin. Experiment 1: samples were lyophilized from 100 μL of 0.05 M TDCl containing varying ratios of D_2O/H_2O at a specific activity of 2843 dpm/nmol of D + H. Back-exchange with atmospheric water vapor occurred for 2 h before sealing in vacuo for decarboxylation. Experiment 2: dry samples were exchanged in vacuo for 3 h at 23 °C over frozen 0.05 M TDCl (1455 dpm/nmol of D + H) and sealed immediately with a torch before heating at 110 °C. Alkaline hydrolysis of a portion of each sample was used to determine the moles of Gla decarboxylated. The total incorporated disintegrations per minute was measured after four lyophilizations from 0.05 M HCl, hydrolysis in 6 M HCl at 100 °C for 24 h, and flash evaporation.

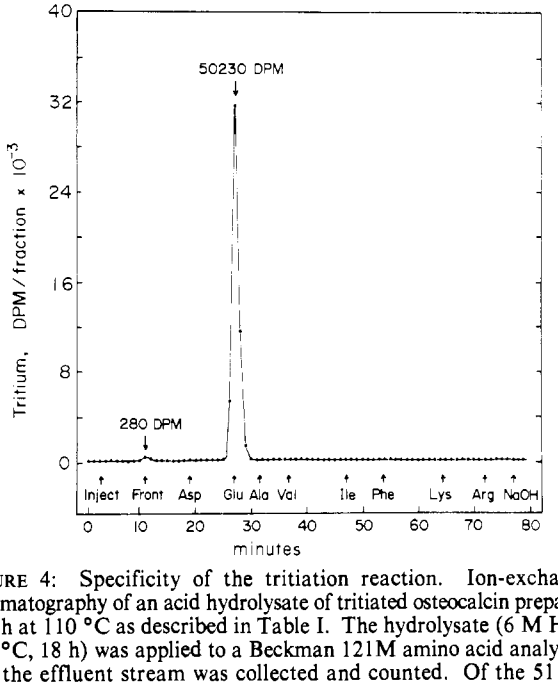

FIGURE 4: Specificity of the tritiation reaction. Ion-exchange chromatography of an acid hydrolysate of tritiated osteocalcin prepared in 3 h at 110 °C as described in Table I. The hydrolysate (6 M HCl, 110 °C, 18 h) was applied to a Beckman 121M amino acid analyzer, and the effluent stream was collected and counted. Of the 51 040 dpm applied, 99% was recovered, with 99.4% of the radioactivity eluting at the position of glutamic acid.

amino acid analyzer. Identical results were obtained on five separate runs using two analyzers with very different buffer programs and elution times for glutamic acid. Alkaline hydrolysates of the labeled protein were also studied, and in these samples ~93% of the radioactivity was in glutamic acid, with the remaining 7% eluting at the column front (a position shared by water and pyroglutamic acid). No evidence of tritiated Gla was found in the alkaline hydrolysates.

A major advantage in labeling Gla-proteins by the present procedure is that the protein remains unchanged except for the substitution of tritiated glutamic acid residues at the positions formerly occupied by Gla. Thus, it is possible to

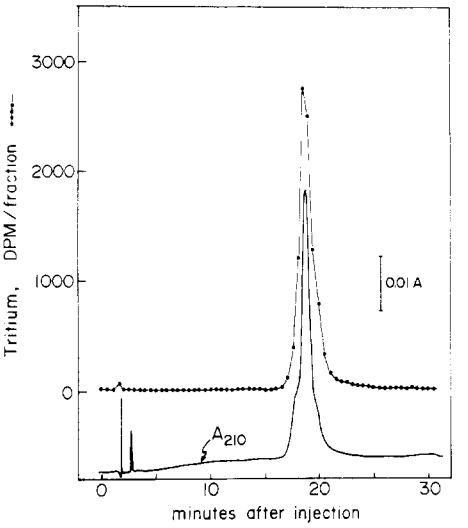

FIGURE 5: High-performance liquid chromatography of the tritium-labeled decarboxylated osteocalcin. Labeled protein (3 h at 110 °C after lyophilization from 0.05 M TDCl; sp act. 5690 dpm/nmol of D) was dialyzed against 0.05 M NH_4HCO_3 and then lyophilized. Eight micrograms of protein was dissolved in 0.01 M triethylamine–formate buffer, pH 4.40, and resolved on a μBondapak-CN column using a 30-min linear gradient of acetonitrile (10–35%) at 2 mL/min. Absorbance was monitored at 210 nm (—). The effluent stream was collected in 1-mL fractions and counted by liquid scintillation (●). Recovery of applied protein was 90%.

prepare high specific activity tritiated, decarboxylated osteocalcin and then study the protein by chromatography. Figure 5 shows the elution profile of osteocalcin on a reverse-phase high-performance liquid chromatography column. The elution is slightly earlier than that of the native protein (data not shown), in agreement with the reduced negative charge of the decarboxylated species [see also Poser & Price (1979)], but otherwise the protein is well-behaved and is inseparable from the incorporated radioactivity.

Discussion

Exchangeable Hydrogens in Osteocalcin. Linderstrøm-Lang and his students thoroughly characterized the classes of exchangeable hydrogen atoms in proteins. They showed that hydrogens bonded to oxygen, nitrogen, and sulfur atoms of amino acid side chains are "instantaneously" exchangeable (≪1 min) with solvent protons (Hvidt & Linderstrøm-Lang, 1955). Amide hydrogens of the peptide backbone are more slowly exchangeable and show both pH-dependent (Hvidt, 1964; Hvidt & Nielsen, 1966; Klotz & Frank, 1965) and conformation-dependent (Lenormant & Blout, 1953; Hvidt, 1955; Willumsen, 1967; Segal & Harrington, 1967) rates of exchange.

Osteocalcin purified from chicken bone is a 6500-dalton peptide containing ~57 amino acid residues (Hauschka & Gallop, 1977). Of the 100 exchangeable hydrogens at pH 1, 49 are in the peptide backbone and 51 are associated with amino acid side chains. Under the conditions of exchange with 0.05 M TDCl (10 min at 20 °C), all of the side-chain atoms and a fraction of the peptide hydrogens should be exchanged with T and D (II in Figure 1). After removal of excess solvent by lyophilization, osteocalcin should contain between 51 and 100 T/molecule. However, there is a lively exchange of hydrogens between atmospheric water vapor and dry proteins (Hvidt & Linderstrøm-Lang, 1955) which is initiated at the instant the lyophilization system is opened. During some of the present experiments, atmospheric humidity was accessible to the dry tritiated protein for up to 2 h before sealing in vacuo

for thermal decarboxylation. The opportunity for loss of T at this step explains why only 32 T are associated with the protein (Table I). Also, this uncontrolled replacement of T (and D) by H at the exchangeable sites of Gla effectively dilutes the specific activity of T and thus lowers the efficiency of tritiated glutamic acid formation below the theoretical value (see below and see Table III).

Once the tritiated protein has been thermally decarboxylated, all unincorporated, exchangeable T must be removed as shown in Figure 1 and Table I. This is achieved by repeated lyophilization and dissolution in 0.05 M HCl. The reluctance of certain peptide backbone T to exchange out is presumably due to conformational restraints discussed above.

Equilibrium Isotope Effects. Preferential fractionation of tritium by the protein during the initial exchange-in procedure would obviously bias the values for stoichiometry of tritium incorporation. However, studies by Englander (1963) and Praissman & Rupley (1968) indicate that there is negligible deviation from a truly random distribution of tritium atoms between the solvent and exchangeable sites on proteins.

Primary (Kinetic) Isotope Effect. The rationale for performing this specific tritium-labeling reaction in deuterated rather than protonated proteins was based on the prediction that primary isotope effects are usually minimizable in this fashion. There are significant effects of atomic mass on both the zero-point energy and vibrational levels of the bonds involving isotopes of hydrogen. Consequently, activation energies are highest for tritium, compared to deuterium and hydrogen, and the rate of reaction is correspondingly reduced for the tritiated compound (Streitwieser, 1960). Reactions involving transfer of the bonding of an isotopic hydrogen from one atom to another typically show very large primary isotope effects, where the rate ratio for the deuterium–hydrogen case (k_H/k_D) can be as much as 7–10 (Shiner et al., 1960). Thus, the efficiency of tritium incorporation is expected to be improved by carrying out the reaction on the protein equilibrated with tritiated 0.05 M DCl compared to using tritiated 0.05 M HCl. When this was done, the improvement was rather modest (38%), as shown in Table III.

Pathway for Tritium Incorporation by Thermal Decarboxylation. Thermal decarboxylation of Gla residues is an unusual reaction which probably precludes the occurrence of primary (kinetic) isotope effects. The reason for this lies in the source of the proton which forms a bond to the γ carbon of Gla during decarboxylation. Possible candidates are (1) the departing γ-carboxyl group, (2) the remaining γ-carboxyl group, (3) other exchangeable hydrogens on adjacent amino acid side chains, (4) peptide hydrogens, or (5) tightly bound water. It is also probable that the acidic labeling conditions allow exchange of T directly with the γ-carbon hydrogen of Gla in the initial step of Figure 1. Mass spectrometry studies show this atom to be rather labile (Stenflo et al., 1974). Subsequent decarboxylation would immediately "fix" this T into glutamic acid. In cases where only *one* group can provide the second proton, the efficiency of T incorporation should theoretically be 2.00 mol of T per mol of Gla decarboxylated. However, if there were *several* possible competing proton donors, then kinetic isotope effects would come into play and the labeling efficiency would decrease to a level determined by the ratios k_D/k_T and k_H/k_T.

Explanation of Reduced Labeling Efficiency. In the first several experiments, the measured efficiency of tritium incorporation into newly formed glutamic acid residues ranged from 0.068 to 0.206 mol of T per mol of Gla decarboxylated (Table II, Figure 3). For osteocalcin, most values clustered around 0.16. This was initially interpreted as a kinetic isotope effect because of the deviation from the expected value of 2.00. However, the measured efficiency was changed only slightly when similar experiments were performed with osteocalcin dried from 0.05 M TDCl in 90% H_2O–10% D_2O rather than from 0.05M TDCl in 99.7% D_2O (expt 1 in Table III).

The lack of a major kinetic isotope effect in these experiments reveals the true cause of the subtheoretical labeling efficiency: namely, that rapid vapor-phase exchange of side-chain hydrogens (H, D, and T) occurs between the dry, tritiated protein and atmospheric water vapor. Thus, the specific activity of T in the dry protein is actually reduced rather rapidly before the tubes are sealed in vacuo for thermal decarboxylation. Studies by vacuum line isolation techniques confirmed that such a back-exchange reaction occurs for tritiated osteocalcin with atmospheric water vapor. The rate of this type of back-exchange reaction can be very high; for insulin, Hvidt & Linderstrøm-Lang (1955) found that all the exchangeable side-chain hydrogens would exchange with ambient water vapor in the typical laboratory atmosphere with a half-time of ~10 min at 25 °C. No special precautions were taken to prevent back-exchange of this type for osteocalcin, except as shown in expt 2 of Table III. This experiment shows that when osteocalcin is equilibrated with 0.05 M TDCl vapor and then immediately sealed from the vapor system with a torch, the efficiency of tritium labeling jumps to 0.95 ± 0.14 mol of T per mol of Gla decarboxylated.

Possible Routes for Background Tritium Incorporation by Non-Gla-proteins. The various possibilities include (1) exchange into very slowly exchanging classes of peptide hydrogens, (2) exchange with certain hydrogens of amino acid side chains which have become labile because of unusual side reactions which accompany the in vacuo heating at 110 °C, and (3) reverse aldol decomposition of serine and threonine to glycine. It is expected that even the most recalcitrant peptide hydrogens would be removed by the final hydrolysis in 6 M HCl at 110 °C. Regarding the second possibility, however, Battersby & Robinson (1955) showed that glutamic and aspartic acid residues in peptides can cyclize to form aminoglutarimide and succinimide congeners. Pyroglutamic acid may also form depending on which nitrogen atom of the peptide backbone is attacked to form the five-membered ring. Similarly, Gla residues could cyclize to form pyro-Gla (Hauschka et al., 1976) or 3-carboxy-5-aminoglutarimide in peptide linkage. In the cyclized form, hydrogen(s) attached to the γ carbon should be more exchangeable, possibly providing a route for nonspecific tritium incorporation, although strong base catalysis would probably be required to withdraw the proton. The contribution of these cyclization side reactions to the overall level of tritium incorporation appears to be minimal for the following reasons: (1) no tritiated glutamic or aspartic acid is found in acid hydrolysates of non-Gla-proteins (Table II), and (2) no tritiated Gla is found in any Gla-proteins after alkaline hydrolysis. The small amount of nonvolatile radioactivity in acid hydrolysates of tritiated non-Gla-proteins (Table II) actually appears to elute in the region of glycine and alanine. Reverse aldol reactions of serine and threonine could occur slowly at 110 °C, resulting in the formation of tritiated glycine.

It is possible that the labeled glutamic acid found in acid and alkaline hydrolysates of tritiated Gla-proteins is actually present as tritiated pyroglutamic acid in the intact polypeptide chain. This would certainly compromise the use of such peptides as substrates for the vitamin K dependent carboxylase system. The possible existence of pyroglutamic acid is being

investigated by permethylation techniques.

The presently described technique for labeling Gla residues may find some application in the sensitive detection of this amino acid. Theoretically, one could achieve a specific activity of $\sim 6 \times 10^4$ dpm/pmol of Gla decarboxylated by using *pure* T_2O as the exchange solvent. However, there is sensibly a practical limit imposed by the specific activity of commercially available T_2O, namely, ~ 10 dpm/pmol of Gla decarboxylated. This level of radioactivity offers no significant advantage over the quantitative detection of Gla by ninhydrin methods (Hauschka, 1977).

Many uses for high specific activity, selectively tritiated Gla-proteins are evident. We are using the tritiated osteocalcin as a substrate for proteolytic digestion and identification of Gla-peptides. Sequencing of these peptides will be greatly facilitated by the discrete pattern of labeled glutamic acid residues at former Gla positions. Mass spectrometric sequence studies would be aided because of a 2-unit mass increase in the glutamic acid residues produced by decarboxylation of Gla after equilibration with 0.05 M DCl or 0.05 M TDCl. We are exploring the possibility of using these peptides as substrates for the bone carboxylase system where vitamin K dependent carboxylation could be studied by tritium release rather than by $^{14}CO_2$ incorporation. Important new information about the mechanism of vitamin K dependent carboxylation has already been obtained by Friedman et al. (1979b) using such methods. Tissue specific distribution of tritiated proteins after injection in vivo may provide some clues regarding function. Finally, it should be possible to carry out specific in situ tritiation of Gla-proteins in histologic sections of virtually any tissue and thereby localize Gla-proteins by autoradiography at the light and electron microscopic levels.

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Physical–Chemical Characterization and Carbohydrate-Binding Activity of the A and B Subunits of the *Bandeiraea simplicifolia* I Isolectins†

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ABSTRACT: *Bandeiraea simplicifolia* I plant seed isolectins comprise a family of tetrameric α-D-galactopyranosyl-binding glycoproteins composed of various combinations of two different kinds of subunits designated A and B. Subtypes of the A (A^a, A^b, A^c, A^d, and A^e) and B (B^a, B^b, B^c, B^d, and B^e) subunits were demonstrated by isoelectric focusing in 8 M urea. Although the content of subunit subtypes varies from seed to seed (e.g., some seeds contain only B subunits, others only A subunits), subtypes A^c and B^c predominate in a natural mixture of the isolectins. Two-dimensional agar gel diffusion studies indicate that, in addition to common structural features, each subunit contains its own distinct antigenic determinants. Although the A and B subunits have closely similar amino acid compositions, they differ markedly in one respect: the B subunit has one methionine residue whereas the A subunit contains no methionine. The neutral carbohydrate content of both subunits is identical. The ability of biopolymers and synthetic glycoproteins to precipitate A_4 and B_4, as well as the capacity of sugars and oligosaccharides to inhibit precipitate formation, was examined. On the basis of these studies, it is suggested that hydrogen bonding occurs between the *hydrogen* atoms of the C-3 and C-4 hydroxyl groups of α-D-GalNAc*p* and α-D-Gal*p* units and the A and B subunits, respectively.

The seeds of *Bandeiraea simplicifolia* contain at least three distinct species of lectins. These are a family of α-D-galactopyranosyl-binding isolectin (BS I) (Murphy & Goldstein, 1977), an *N*-acetyl-D-glucosamine-binding lectin (BS II) (Shankar Iyer et al., 1976), and a family of *N*-acetyl-D-galactosamine binding isolectins (BS III) (Murphy, 1978). The family of α-D-galactose-binding isolectins contains five principle isolectin forms: BS I A_4, A_3B, A_2B_2, AB_3, and B_4 which are tetrameric structures composed of two major glycoprotein subunit types designated A and B (Murphy & Goldstein, 1977). A detailed description of the binding of the BS I isolectins to human red blood cells has been reported (Judd et al., 1978). An earlier report on the carbohydrate-binding specificity of the two subunits of the BS I isolectins indicated that the A subunit exhibited a primary specificity for α-D-GalNAc but also cross-reacted with α-D-Gal*p* whereas the B subunit showed a sharp specificity for α-D-Gal*p* residues (Murphy & Goldstein, 1977). In this communication we extend the comparative studies on the carbohydrate-binding sites of the two extreme forms of the BS I isolectins (A_4 and B_4) and report on the physiochemical properties of the BS I isolectins.

Experimental Section

Materials

Sugars and Sugar Derivatives. Me-α-D-GalNAc was prepared by the procedure of Neuberger & Wilson (1971). Methyl 4-deoxy-4-fluoro-α-D-galactopyranoside was prepared by the procedure of Marcus & Westwood (1971). Dr. R. U. Lemieux, University of Alberta, donated 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, 6-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, and 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-glucose. *p*-NO_2Ph-α-D-GalNAc*p* was a gift from Dr. B. Weissmann, University of Illinois. Methyl 4-*O*-(α-D-galactopyranosyl)-α-D-galactopyranoside was donated by Dr. E. J. Reist, SRI International, Menlo Park, CA. 1-*O*-(6-*O*-α-D-Galactopyranosyl-1-*O*-β-D-galactopyranosyl)-D-glycerol was a gift from Dr. D. Myhre, Procter and Gamble Co. *p*-NO_2Ph-β-D-GalNAc and *p*-NO_2Ph-α- and -β-D-Gal*p* were obtained from Cyclo Chemical Co., Los Angeles, CA; 2-deoxy-D-galactose was obtained from P-L Biochemicals, Milwaukee, WI. Methyl β-D-thiogalactoside was obtained from Vega-Fox-Biochemicals, Tuscon, AZ. All other sugars were obtained from Pfansteihl Laboratories, Waukegan, IL. D-GalNAc obtained from Pfanstiehl required filtration through a PM-10 membrane (Amicon Corp.) before use.

Glycoproteins and Polysaccharides. Type B ovarian cyst substance was kindly provided by Dr. A. Lundblad of the University of Lund; type A cyst substance and hog A + H mucin were provided by Dr. R. D. Poretz, Rutgers University. The galactomannan (guaran) of *Cyamopsis tetragonolobus* was purified as described by Hayes & Goldstein (1974). The arabinogalactan from *Larix occidentalis* was a gift of Professor B. Lindberg of the University of Stockholm. The carbohydrate–bovine serum albumin conjugate *p*-azophenyl β-D-lactoside–BSA was available from an earlier study in this laboratory (Iyer & Goldstein, 1973). The *p*-azophenyl-*N*-acetyl-α- and -*N*-acetyl-β-D-galactosamine–BSA and the *p*-azophenyl α- and β-D-galactopyranoside–BSA conjugates were prepared similarly. All carbohydrate–protein conjugates

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