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Kinetics of Intracellular Degradation of Newly Synthesized Collagen†

Robert S. Bienkowski*

Pulmonary Division, Department of Pediatrics, Schneider Children's Hospital of Long Island Jewish Medical Center, New Hyde Park, New York 11042

Samantha F. Curran and Richard A. Berg

Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, New Jersey 08854

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ABSTRACT: The objective of this work was to determine the time dependence of the basal component of intracellular degradation of newly synthesized collagen. Chick embryo tendon fibroblasts were incubated with [^{14}C]proline, and degradation was quantified by measuring hydroxy[^{14}C]proline in a low molecular weight fraction. When cultures were pulse labeled for 15 min and then incubated under chase conditions for 105 min, the amount of degraded collagen attained a value equal to approximately 20% of the amount synthesized during the labeling period; the data were fit with a simple exponential function that had a 40-min rise time and a 12-min lag time. In continuously labeled cultures, the rates of collagen synthesis and secretion reached constant values within 15 and 45 min, respectively. Degradation products were first detected 6–9 min after collagen synthesis began and were transported out of the cells more rapidly than intact collagenous molecules; however, percent degradation increased slowly and did not reach a constant value even after 240 min of incubation. Since collagen degradation lags collagen synthesis, it follows that degradation is a posttranslational, rather than a cotranslational, process, and since degradation and secretion are kinetically distinguishable, it follows that they occur in parallel pathways. A simple nonlinear model for posttranslational processing of collagen is proposed.

Although many secretory proteins are catabolized soon after being synthesized rather than transported intact to the extracellular space (Bienkowski, 1983), rapid intracellular degradation has been documented most extensively for collagen synthesized by connective tissue cells in culture (Rennard et al., 1982; Bienkowski, 1984a). Recent work has shown that catabolism of newly synthesized collagen can proceed by at least two different pathways (Berg et al., 1980; Bienkowski, 1984a). One of these, the basal pathway, accounts for the breakdown of 10–20% of all newly synthesized collagen (Bienkowski, 1984b). Another pathway is activated when structurally abnormal collagen is synthesized; abnormalities in molecular structure can be caused by depriving cultures of ascorbate or by adding a proline analogue to the incubation medium. Several lines of evidence demonstrate that enhanced degradation is mediated by lysosomal proteases and occurs in lysosomes (Berg et al., 1980, 1984; Bienkowski, 1984b). In contrast, little is known about the mechanism of basal degradation. A complete description of this pathway would require identification of its constituent proteases and substrates, determination of its location within the cell, and measurement of the time scale on which it operates relative to synthesis and posttranslational processing of collagen.

The specific questions we wished to address in the present study were the following: When does basal degradation begin, and what is the time dependence for the generation of degradation products? We used matrix-free tendon fibroblasts from chick embryos because a large percentage of the protein synthetic activity in these cells is devoted to collagen and because the kinetics for the synthesis, posttranslational modification, and secretion of collagen have been established for these cells (Dehm & Prockop, 1972; Harwood et al., 1976; Kao et al., 1977, 1979). Our data show that, in comparison to synthesis and secretion of collagen, intracellular degradation is a very slow process.

Experimental Procedures

Materials. Radioactive isotopes were purchased from New England Nuclear, Boston, MA. L-[U-^{14}C]Proline (approximate specific activity 280 mCi/mol) had a stated radiochemical purity >99%, and this was verified in our laboratory. *trans*-4-Hydroxy-L-[G-^{3}H]proline (specific activity 5.4 Ci/mol) was purified by ion-exchange chromatography as described (Bienkowski & Engels, 1981). Purified bacterial collagenase (form III) was purchased from Advance Biofactures, Lynbrook, NY.

Incubation Protocols. Tendon fibroblasts were isolated from 17-day-old chick embryos according to the method of Dehm and Prockop (1972) as modified by Kao et al. (1977). The cells were suspended at a density of approximately 10^7/mL

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* Author to whom correspondence should be addressed.

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in Krebs II buffer supplemented with ascorbic acid (10 μg/mL). In the continuous labeling experiments, cells were incubated at 37 °C with [^{14}C]proline (10 μCi/mL) for 0–240 min. In the pulse–chase experiment, cells were incubated with [^{14}C]proline (10 μCi/mL) for 15 min, then the cell suspension was centrifuged, and the cells were suspended in Krebs buffer containing 1 mM unlabeled proline; incubation continued for 0–120 min. All incubations were done in triplicate. Depending on the objectives of the experiment, the cells and medium were either processed separately or processed as a combined sample.

Analytical Techniques. Percent collagen production, that is, the percentage of total protein production which is devoted to collagen, was determined by the collagenase digestion technique described by Peterkofsky and Diegelmann (1971) and modified by Berg et al. (1981); a correction was made for the greater abundance of proline in collagen than in other proteins (Breul et al., 1980).

To study intracellular degradation of collagen, hydroxy-[^{14}C]proline-containing peptides were separated into low and high molecular weight peptides according to whether they were soluble or insoluble in cold 0.15 N NaCl/ethanol (1:3). A known amount of hydroxy[^{3}H]proline (~10^6 dpm) was added to each sample as a recovery standard, and the samples were hydrolyzed (6 N HCl, 110 °C, 24 h). Each alcohol-soluble sample was chromatographed on a column of AG 50-X8 resin to seperate the hydroxyprolines from the [^{14}C]proline; then those fractions containing hydroxy[^{3}H]proline were combined and desalted with AG 2-X8 resin. A column of an analytic cation-exchange resin (DC6A, Dionex) was used to quantitate hydroxy[^{14}C]proline and hydroxy[^{3}H]proline. Details of the chromatography systems have been published (Bienkowski & Engels, 1981). The absolute amount of hydroxy[^{14}C]proline in each sample was calculated by multiplying the ^{14}C/^{3}H ratio by the quantity of hydroxy[^{3}H]proline added to the sample. The extent of collagen degradation was measured by determining the ratio of hydroxy[^{14}C]proline in the soluble fraction to total hydroxy[^{14}C]proline; letting S designate the hydroxy[^{14}C]proline in the soluble fraction and P the hydroxy-[^{14}C]proline in the insoluble fraction, the percent degradation, D, is

$$D = 100[S/(P + S)] \quad (1)$$

It is to be emphasized that D characterizes the entire system rather than the cells or the medium separately. In those experiments in which the compartments were analyzed separately, the quantities $S = S_{cells} + S_{medium}$ and $P = P_{cells} + P_{medium}$ were used to calculate the percent degradation.

Results

Time Scales for Production and Secretion of Collagen. The incorporation of [^{14}C]proline into collagenase-sensitive material (high molecular weight collagenous peptides) is shown as a function of labeling time in Figure 1A; the rate of incorporation was constant for at least 100 min, and the lag time was very short, approximately 9 min. Percent collagen production for the system (cells plus medium) reached a constant value of approximately 23% between 15 and 30 min after the start of labeling (Figure 1B); within the cells, a constant value of 23% was reached by 15 min, while in the medium the steady-state level of approximately 27% was reached after 45 min. The distribution of newly synthesized collagenous peptides between cells and medium is shown as a function of labeling time in Figure 1C. Collagenous polypeptides were first detected in the medium between 15 and 30 min. The slope of the curve, which is proportional to the rate of secretion, attains a constant value after approximately 60 min; the lag

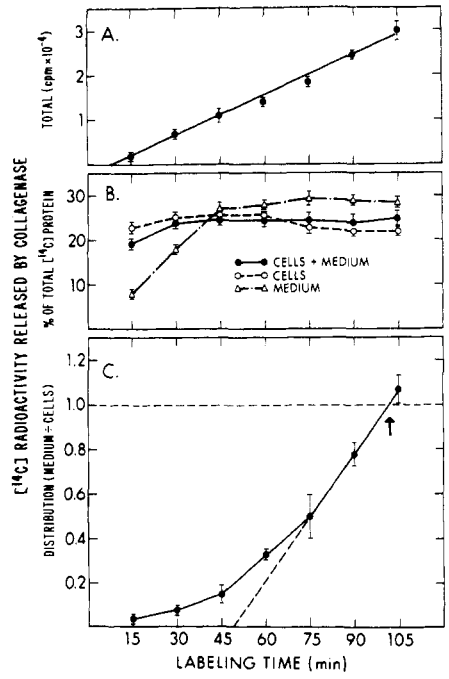

FIGURE 1: Collagen production in matrix-free cells. (A) Incorporation of [^{14}C]proline into collagenase-sensitive material as a function of labeling time. Each point is the average of three determinations, and the error bars represent ±1 SD. The straight line is a least-squares fit to the data; the line intersects the time axis at 9 min. (B) Percent collagen production as a function of labeling time. The cells and medium were analyzed separately. (C) Distribution of newly synthesized collagenase-sensitive protein between medium and cells. The ratio of the amount in the medium to the amount in the cells is shown as a function of labeling time; the ratio is 1 at approximately 100 min (arrow). The dashed line extrapolated from the straight line connecting points at 75, 90, and 105 min intersects the time axis at 48 min.

time, the point at which the extrapolated straight line intersects the time axis, is approximately 45 min. During the first 100 min of labeling, the amount of radiolabeled collagen in the medium was less than the amount within the cells.

Earliest Appearance of Degradation Products. A series of experiments was undertaken to determine how soon collagen degradation products could be observed after metabolic labeling began. The major factor limiting the ability to detect hydroxy[^{14}C]proline in the low molecular weight fraction was the uncertainty in the background level of ^{14}C radioactivity. The magnitude of this uncertainty was measured by processing triplicate "blank" samples of [^{14}C]proline in the same way as the incubated samples; the average amount of ^{14}C radioactivity that coeluted with hydroxy[^{3}H]proline was typically 2000 ± 500 dpm (mean ± range) per 10 μCi of [^{14}C]proline. Thus, the minimum amount of metabolically produced hydroxy-[^{14}C]proline that could be detected reliably was approximately 500 dpm above the average background. Figure 2A illustrates this point; the elution profile for an alcohol-soluble fraction from a culture incubated with 10 μCi of [^{14}C]proline for 30 min is superimposed on a band defined by the elution profiles of three 10-μCi samples of isotope. The contribution of background radioactivity in the low molecular weight fraction was insignificant when long labeling times (e.g., 120 min) were used (Figure 2B), and there was little difficulty in quantitating hydroxy[^{14}C]proline in the high molecular weight (alcohol-insoluble) fraction when either short or long labeling times were employed (Figure 2C,D). It should be noted that the [^{14}C]proline used in this work was of very high radiochemical purity and attempts to attain lower background levels by subjecting the isotope to preparative ion-exchange chromatography were unsuccessful (Bienkowski, 1984c).

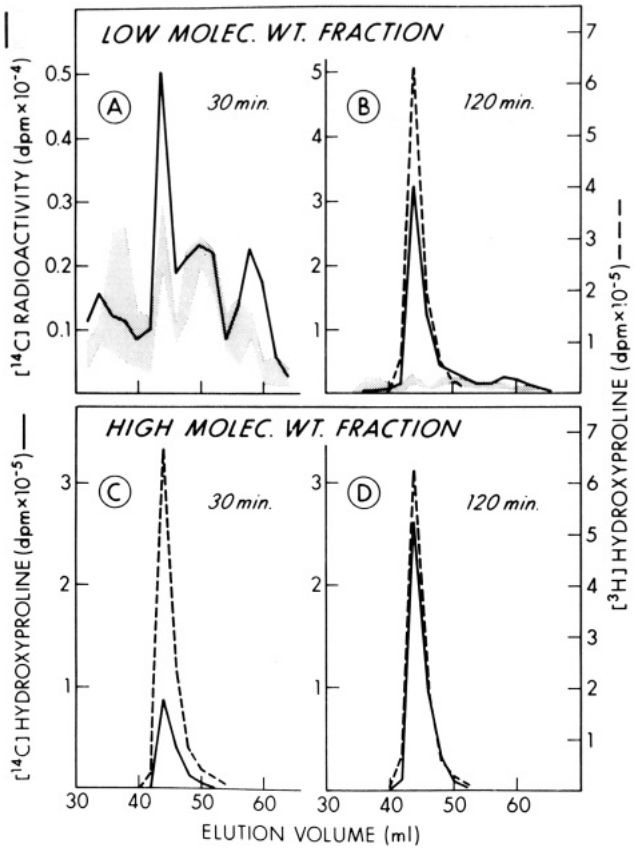

FIGURE 2: Analysis by cation-exchange chromatography of hydroxy[^{14}C]proline produced by matrix-free cells incubated with [^{14}C]proline for either 30 or 120 min. The low and high molecular weight fractions contain molecular species that are respectively soluble and insoluble in cold 0.15 N NaCl/ethanol (1:3). The shaded bands in (A) and (B) represent the background radioactivity contributed by the [^{14}C]proline. The dashed curve in panels B–D indicates the elution profile of hydroxy[^{3}H]proline which was added to each sample as an internal recovery standard; for clarity it is not shown in panel A.

In an experiment employing short labeling times, degradation products were not detectable at 6 min; they were barely detectable after 12 min (200 ± 100 dpm above the upper limit of the background range), and they were easily detectable after 18 min (1200 dpm above background range). In several other experiments, small amounts of hydroxy[^{14}C]proline could be consistently detected in the alcohol-soluble fraction 15 min after incubation with [^{14}C]proline commenced. Hydroxy-[^{14}C]proline was found in the alcohol-insoluble fraction at all times studied. Thus, there was a lag of approximately 6–9 min between the earliest time hydroxy[^{14}C]proline appeared in the high molecular weight fraction and the time it appeared as a degradation product.

Time Course of Collagen Degradation. The time course for generation of degradation products in two separate continuous labeling experiments is shown in Figure 3A. The curves are nonlinear over the time span of the experiments (0–90 min), suggesting that the rate of degradation did not attain a constant value for at least 75–90 min. The distribution of the degradation products between cells and medium (Figure 3B) differs markedly from the distribution of collagenase-sensitive material (Figure 1C). Note that most of the small hydroxy[^{14}C]proline-containing peptides were within the cells for 60–75 min, which is consistent with their being generated intracellularly, whereas most of the high molecular weight hydroxy[^{14}C]proline-containing peptides were within the cells for approximately 100 min (see Figure 1C).

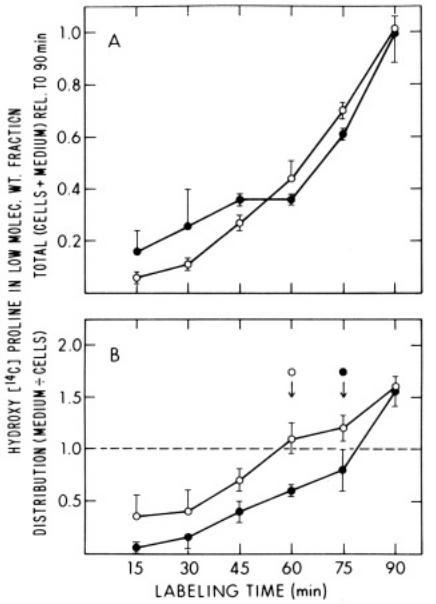

FIGURE 3: Generation of collagen degradation products. (A) Hydroxy[^{14}C]proline in the low molecular weight fraction vs. labeling time for two independent experiments. Because of small differences in the amounts of isotope and numbers of cells per milliliter used in the experiments, data are expressed relative to the amount of hydroxy[^{14}C]proline in the low molecular weight fraction at 90 min [(○) 3×10^4 dpm; (●) 2.6×10^4 dpm]. (B) Distribution of the degradation products between medium and cells. The ratio of hydroxy[^{14}C]proline in the medium to hydroxy[^{14}C]proline in the cells is shown as a function of labeling time for two independent experiments; the arrows indicate the time when the ratio equals 1 for each experiment [(○) 60 min; (●) 75 min].

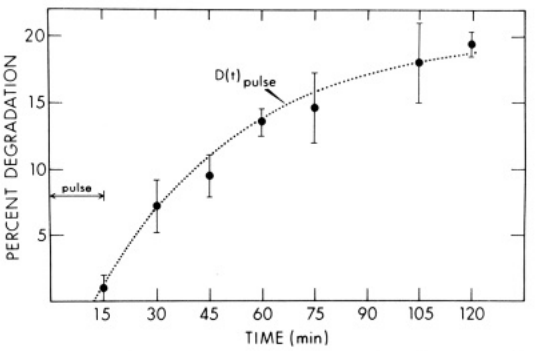

FIGURE 4: Percent degradation vs. time in a pulse–chase experiment. The pulse period was 15 min. Each experimental point is the average of three determinations, and the error bars represent ±1 SD. The dotted curve, $D(t)_{pulse}$, was calculated by using eq 2.

The time course of percent intracellular degradation was studied by using both pulse–chase and continuous labeling protocols. Figure 4 shows results for an experiment in which cultures were pulse labeled for 15 min and then incubated under chase conditions for 0–105 min. Degradation was approximately 1% at the beginning of the chase and approximately 20% at the end of the chase. Regression analysis was used to fit the experimental data with the following simple exponential function of time:

$$D(t)_{pulse} = 20[1 - e^{-k(t-12)}] \quad (2)$$

where $k = 0.025$ min^{-1}. The correlation coefficient for the regression of ln (20 − D) vs. time is $r = 0.98$. Equation 2 is not valid for times less than 12 min, which represents a lag between the start of labeling and the appearance of degradation products.

The time course of intracellular degradation in several continuous labeling experiments is shown in Figure 5. Al-

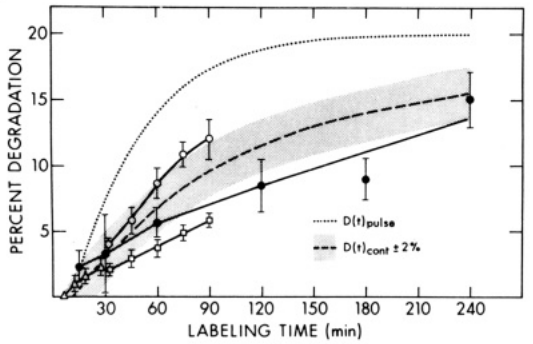

FIGURE 5: Percent degradation vs. time in continuous labeling experiments. The results of five independent experiments are shown; each data point is the average of three determinations, and the error bars indicate ±1 SD. The dashed curve, $D(t)_{cont}$, was calculated by using eq 3; the shaded band represents an uncertainty of ±2% in the calculated value.

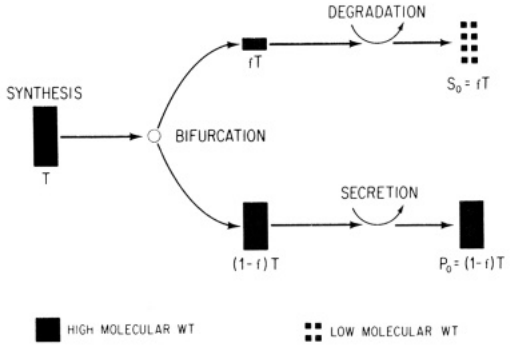

FIGURE 6: Kinetic model for intracellular degradation of newly synthesized collagen. At the bifurcation a pulse of high molecular weight collagenous material, T, is partitioned between the degradation and secretion pathways in the ratio $f/(1-f)$, where f, the partition coefficient, is <1. The final amount of degradation product is $S_0 = fT$, and the final amount of intact, secreted collagen is $P_0 = (1-f)T$.

though there are considerable differences among the curves generated in independent incubations, they have several common features: All are below the curve fit to the pulse–chase data, $D(t)_{pulse}$; none reaches the high level of degradation (20%) observed in the pulse–chase experiment; none attains a constant value. The time-averaged integral of eq 2 yields a predicted curve, $D(t)_{cont}$, for the continuous labeling experiments:

$$D(t)_{cont} = 20\{1 - [1 - e^{-k(t-12)}]/[k(t-12)]\} = 20 - [D(t)_{pulse}/[k(t-12)]] \quad (3)$$

This curve and a band which represents a ±2% uncertainty in measuring degradation are also shown in Figure 5. The function $D(t)_{cont}$ approximates an "average" of all the experimental data, and most of the individual data points fall within the error band.

In considering the data presented in Figures 3A, 4, and 5, it is important to distinguish between rate of degradation and extent of (or percent) degradation: Constant percent degradation implies a constant rate of degradation; this condition defines the steady state of the degradation pathway, and it is approached when the time is very long compared to the time constant of the system ($t \gg k^{-1}$). In a pulse–chase experiment the steady-state rate of degradation is zero, whereas in a continuous labeling experiment the rate is greater than zero. Although the steady-state values of percent degradation are the same for eq 2 and 3, viz., 20%, $D(t)_{pulse}$ rises more rapidly than $D(t)_{cont}$, which agrees with the experimental observations made on the pulsed and continuously labeled systems.

It is not clear why the time course of intracellular degradation varied so much among the different continuous labeling experiments; however, two points suggest that the manifestation of intracellular degradation is subject to considerable biological variation. First, as illustrated in Figure 5, intraexperimental error in quantitating percent degradation was usually less than interexperimental variability; second, while the time courses for *generation* of degradation products in two independent experiments were similar (Figure 3A), the time courses for *secretion* of the degradation products were quite different (Figure 3B). The variability between experiments does not affect the major conclusions to be drawn from this study.

Synthesis, Degradation, and Production of Collagen. Net collagen production, i.e., the amount of collagen present at any given time, is determined by the opposing effects of synthesis and degradation. As already noted, Figure 1A shows the time course of production in a continuous labeling experiment; the amount of collagen synthesized is the sum of the amount degraded and the net amount produced. For example, at 105 min the average degradation is approximately 10% (Figure 5) and the amount synthesized exceeds the amount produced by approximately 11%. However, as the error bars in Figure 1A indicate, the uncertainty in the measurement of production is approximately 2 parts in 30, or ±7%. Therefore, over the time span of this experiment it is difficult to distinguish synthesis and net production directly, and Figure 1A can be considered to show, in effect, the time course of collagen synthesis.

Discussion

When matrix-free chick embryo tendon fibroblasts were continuously incubated with [^{14}C]proline, synthesis of radioactively labeled collagen attained a constant rate within 10–15 min (Figure 1A,B), and the rate of secretion of collagen from the cells to the medium reached a constant value by 45 min (Figure 1B,C). In comparison to synthesis and secretion, degradation is a slow process: The appearance of degradation products lagged the appearance of collagen by 6–9 min, and the extent of degradation did not reach a steady-state level even during labeling periods of up to 240 min (compare Figure 1B and Figure 5). Interestingly, however, once the degradation products were generated, they were transported out of the cells faster than the high molecular weight collagenous peptides (compare Figure 3B and Figure 1C).

Three conclusions can be drawn from these findings. First, since degradation lags both synthesis and secretion, it follows that degradation is not a cotranslational process. Second, the high and low molecular weight hydroxyproline-containing species are transported out of cells by different mechanisms. Third, and most important, because secretion and degradation operate simultaneously but evolve on different time scales, it follows that posttranslational processing of collagen comprises a nonlinear set of operations. This in turn requires the existence of a branch point, or a bifurcation, at which newly synthesized collagen molecules enter one of various pathways to be processed in different ways. A simple model of nonlinear processing is shown in Figure 6. At the bifurcation, a pulse of newly synthesized collagen, designated T, is partitioned between the basal degradation pathway and the secretion pathway in the ratio $f/(1-f)$, where f, the partition coefficient, has a value between 0 and 1 ($0 < f < 1$). The final amount of degradation product is $S_0 = fT$, and the final amount of secreted collagen is $P_0 = (1-f)T$. The partition coefficient determines the final value of degradation; it follows from eq 1 that $D_0 = 100f$. A description of the temporal development of percent degradation requires a knowledge of the rate of generation of degradation products. For simplicity we assume

that this rate is proportional to available substrate in the degradation pathway:

$$dS/dt = k(fT - S) = k(S_0 - S) \quad (4)$$

The solution of this equation is

$$S(t) = S_0[1 - e^{-k(t-t_0)}] \quad (5)$$

Since $T = S_0 + P_0 = S(t) + P(t)$, both sides of eq 5 can be multiplied by $100/T$ to yield

$$D(t)_{model} = D_0[1 - e^{-k(t-t_0)}] \quad (6)$$

This expression is formally identical with eq 2 which was fit to data from the pulse–chase experiment. The reciprocal of the rate constant (k^{-1}) defines a time scale on which the degradation mechanism responds to the substrate, and t_0 can be interpreted as a lag between the start of the experiment and the time that substrate reaches the degradation mechanism. As already noted, the final value of percent degradation, D_0, is determined by the partition coefficient, f, and it is independent of the form of the rate equation. The basic nonlinearity of the model is reflected in the condition that $0 < f < 1$ (which is equivalent to requiring that $0 < D < 100\%$); the model becomes linear in the limiting cases $f = 0$ or $f = 1$.

In summary, the experimental findings presented here and the kinetic model which accounts for the data lead us to conclude that basal degradation of newly synthesized collagen is a posttranslational operation which occurs in a pathway that is kinetically distinct from the pathway leading to secretion. We emphasize, however, that this conclusion does not imply that basal degradation occurs in a subcellular compartment that is morphologically distinct from the sites of other post-translational processes. We are currently pursuing studies to determine the locus of basal degradation.

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Inactivation of D-(−)-β-Hydroxybutyrate Dehydrogenase by Modifiers of Carboxyl and Histidyl Groups†

Paruchuri V. Prasad and Youssef Hatefi*

Division of Biochemistry, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037

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ABSTRACT: Data presented in this paper suggest that D-(−)-β-hydroxybutyrate dehydrogenase (BDH) purified from bovine heart mitochondria contains an essential carboxyl group and an essential histidyl residue at or near the active site. Lactate and malate dehydrogenases, which catalyze reactions analogous to that catalyzed by BDH, also contain an aspartyl and a histidyl residue at the active site [Birktoft, J. J., & Banaszak, L. J. (1983) *J. Biol. Chem. 258*, 472–482]. In addition, all three enzymes contain an essential arginyl residue, apparently concerned with electrostatic interaction with their respective carboxylic acid substrates, and promote ternary adduct formation involving the enzyme, NAD, and sulfite.

D-(−)-β-Hydroxybutyrate dehydrogenase (BDH,[1] EC 1.1.1.30) is a phospholipid-requiring enzyme that is associated with the mitochondrial inner membrane. BDH catalyzes with ordered bi bi kinetics the reversible oxidation of D-(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD. The enzyme has been purified from bovine heart (Bock & Fleischer, 1975) and rat liver (Kebbaj et al., 1982) mitochondria. The bovine enzyme has a monomer M_r of 31 500, and the phospholipid-reconstituted holoenzyme has been reported to be tetrameric (McIntyre et al., 1983) or a mixture of aggregates (Yamaguchi & Hatefi, 1985). BDH contains essential arginyl (Phelps & Hatefi, 1981a; Kebbaj et al., 1982;

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*Author to whom correspondence should be addressed.

[1] Abbreviations: BDH, LDH, and MDH, β-hydroxybutyrate, lactate, and malate dehydrogenases, respectively; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EFA, ethoxyformic anhydride; *p*HMB, *p*-(hydroxymercuri)benzoate; SDS, sodium dodecyl sulfate; His, histidine; Asp, aspartic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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