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Modification of Arginine and Lysine in Proteins with 2,4-Pentanedione†

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ABSTRACT: Primary amines react with 2,4-pentanedione at pH 6–9 to form enamines, *N*-alkyl-4-amino-3-penten-2-ones. The latter compounds readily regenerate the primary amine at low pH or on treatment with hydroxylamine. Guanidine and substituted guanidines react with 2,4-pentanedione to form *N*-substituted 2-amino-4,6-dimethylpyrimidines at a rate which is lower by at least a factor of 20 than the rate of reaction of 2,4-pentanedione with primary amines. Selective modification of lysine and arginine side chains in proteins can readily be achieved with 2,4-pentanedione. Modification of lysine is favored by reaction at pH 7 or for short reaction times at pH 9. Selective modification of arginine is achieved by reaction with 2,4-pentanedione for long times at pH 9, followed by treatment of the protein with hydroxylamine. The extent of modification of lysine and arginine side chains can readily be measured spectrophotometrically. Modification of lysozyme with 2,4-pentanedione at pH 7 results in modification of 3.8 lysine residues and less than 0.4 arginine residue in 24 hr. Modification of lysozyme with 2,4-pentanedione at pH 9 results in modification of 4 lysine residues and 4.5 arginine residues in 100 hr. Treatment of this modified protein with hydroxylamine regenerated the modified lysine residues but caused no change in the modified arginine residues. One arginine residue seems to be essential for the catalytic activity of the enzyme.

In spite of the availability of a variety of reagents for the modification of lysine residues in proteins and a smaller number of reagents for the modification of arginine (Means and Feeney, 1971; Glazer, 1970; Cohen, 1968, 1970), the search for new reagents for the modification of these amino acids continues, with a view toward selective modification and ease of analysis of extent of modification.

The most common modifications of lysine residues in proteins are acylations with anhydrides and arylations with substituted nitrobenzenes (Means and Feeney, 1971). Neither type of modification is entirely specific, because a number of other protein nucleophiles are capable of reacting with these reagents. None of these reactions are at the same time readily reversible and readily quantitatable. In the present paper we show that 2,4-pentanedione reacts rapidly with the side chain amino groups of lysine residues in proteins. No other side chains are rapidly modified, and the extent of modification is readily quantitated spectrophotometrically. Modification can be reversed at neutral pH or by treatment with hydroxylamine.

The modification of the side chains of arginine residues in proteins is more difficult than the modification of other side chain functional groups. 1,2-Dicarbonyl compounds have commonly been used (Patthy and Smith, 1975; Yankeelov, 1972; Takahashi, 1968; Signor et al., 1971) but such modifications often lead to more than one product, produce irreversible modification of lysine side chains, and can be quantitated only by amino acid analysis. Reaction of guanidines with 1,3-dicarbonyl compounds produces pyrimidines in high yield, but in spite of the widespread use of this reaction for the synthesis of pyrimidines (Brown, 1962), it has been little used for the modification of proteins (Beller et al., 1968). In this paper we show that reaction of 2,4-pentanedione with arginine residues of proteins leads to formation of a stable pyrimidine whose presence can be detected spectrophotometrically. The modification of lysine side chains, which also occurs when proteins are treated with this reagent, can easily be reversed following protein modification by treatment with hydroxylamine. The modification of hen egg white lysozyme by 2,4-pentanedione is presented to illustrate the use of this reagent for modification of both lysine and arginine.

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Experimental Section

Materials. 2,4-Pentanedione (Aldrich Gold Label), guanidinium sulfate (Aldrich), arginine hydrochloride (Sigma), *N*-benzoyl-L-argininamide (Sigma), and all inorganic salts were used as received. *n*-Butylamine (Aldrich) was distilled before use. Hen egg white lysozyme (Grade I) and *Micrococcus lysodeikticus* cells were obtained from Sigma. Water was purified by means of a Millipore Super Q water purification system.

Methods. Uv spectra were recorded with a Cary 15 spectrophotometer. Ir spectra were taken on a Beckman IR-8. Nuclear magnetic resonance (NMR) spectra were determined with a Varian A-60. Chemical shifts are given in parts per million downfield from tetramethylsilane. Fluorescence spectra were recorded on an Aminco-Bowman SPF-2 ratio recording spectrophotofluorometer. Kinetic measurements were performed on a Gilford Model 222 photometer attached to a Beckman DU monochromator equipped with a thermostated cell compartment. Amino acid analyses were performed on a Beckman 120C amino acid analyzer using standard techniques. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

N-n-Butyl-4-amino-3-penten-2-one. This compound was prepared by the method of Dudek and Holm (1962).

2-Amino-4,6-dimethylpyrimidine. This compound was prepared by the method of Combes and Combes (1892).

δ-(4,6-Dimethyl-2-pyrimidyl)-N-benzoylornithinamide (**3b**). *N*-benzoylargininamide (1.2 g) was heated for 24 hr at 37° with 1.1 g of K_2CO_3 and 2.0 g of **1** in 30 ml of water. The white precipitate was filtered, washed with cold water and then ethanol, and then recrystallized from ethanol–water: yield, 0.8 g (53%); mp 210–211°; NMR (Me_2SO-*d*; D_2O) 1.70 (4 H, m), 2.12 (6 H, s), 3.22 (2 H, m), 4.10 (1 H, t, J = 7 Hz), 6.28 (1 H, s), 7.42 (3 H, m), 7.85 ppm (2 H, m); ir (KBr) 3400, 3280, 2980, 2960, 1660, 1590, 1550, 1510, 820, 790 cm^{-1}. Mass spectral peak matching sample/reference for $C_{18}H_{23}N_5O_2$: calculated, 1.086645; observed, 1.086644.

δ-(4,6-Dimethyl-2-pyrimidyl)ornithine (**3c**). Arginine hydrochloride (1.0 g), 2.0 g of **1**, and 1.0 g of K_2CO_3 were dissolved in 15 ml of water and the solution was heated at 37° for 32 hr. The pH was then adjusted to 5 with 1 *N* HCl and the solution was kept at 4° for 16 hr. The resulting precipitate was filtered, washed with cold water and then with methanol, and dried under vacuum; 0.31 g (10%) of **3c** was obtained, mp 269–270° (d): NMR (Me_2SO-*d*, D_2O) 1.67 (4 H, m), 2.25 (6 H, s), 3.32 (3 H, m), 6.38 ppm (1 H, s); ir (KBr) 3310, 3000, 1610, 1560, 820, 810, 790 cm^{-1}. Mass spectral peak matching sample/reference for $C_{11}H_{18}N_4O_2$: calculated, 1.08748; found; 1.08747.

Reaction of **2** *with Hydroxylamine.* Enamine **2** (0.5 g) was reacted with 10 g of NH_2OH·HCl in 50 ml of water at pH 6 for 5 min at 21°; 5 ml of concentrated HCl was then added, and the solution was extracted continuously with ether for 48 hr. The ether was dried with Na_2SO_4 and evaporated to dryness. The yellow oil of 4-hydroxy-3-penten-2-one oxime had NMR ($CDCl_3$) 2.22 (3 H, s), 2.38 (3 H, s), 5.80 (1 H, s), and 6.83 ppm (1 H, broad) and ir (neat) 3240, 1600, 1430, and 1250 cm^{-1}. For comparison, the dioxime of **1** was prepared by heating a solution of 1.0 g of **1** with excess NH_2OH in 1 *M* NaOH at 80° for 3 hr. The crystals which resulted on cooling were recrystallized from water: mp (found 155–156°, lit. 152°) (Acly and French, 1927); NMR (Me_2SO-*d*): 1.88 (6 H, s), 3.15 (2 H, s), 10.70 ppm (2 H, s); ir (KBr) 3200, 1660, 1450, 1050, 980 cm^{-1}.

Kinetics of Enamine Formation and Hydrolysis. The formation of enamine **2** was measured spectrophotometrically at 310 nm in 3 ml of a solution containing 0.2 *M* *n*-butylamine, 3.2×10^{-5} *M* **1**, and 0.2 *M* sodium phosphate or sodium carbonate buffer. Enamine hydrolysis was monitored at 310 nm using 2.2×10^{-5} *M* **2** in the same buffers in the absence of *n*-butylamine. For calculation of first-order rate constants, slight adjustments (<10%) of the observed infinity point were used to obtain the most linear plot of log $(A_t - A_\infty)$ vs. time. Following this adjustment, the plots were linear for at least four half-lives. Observed rates of enamine formation were corrected for the rate of enamine hydrolysis before calculation of second-order rate constants.

Kinetics of Formation of 2-Amino-4,6-dimethylpyrimidine. **1** (1×10^{-3} *M*) was reacted with 0.25 *M* guanidinium sulfate in 0.2 *M* sodium phosphate or sodium carbonate buffer at 21°. At various times, a 0.005-ml aliquot was withdrawn and diluted to 1.00 ml, and the fluorescence spectrum of this solution was scanned using an excitation wavelength of 285 nm. Fluorescence emission at 350 nm was used to calculate first-order rate constants.

Assay of Lysozyme. Micrococcus lysodeikticus cells (12 mg) were suspended in 100 ml of 0.066 *M* sodium phosphate buffer (pH 6.24). The decrease in turbidity of 3.0 ml of this solution at 450 nm and 25° was used to measure the activity of lysozyme preparations. Lysozyme concentrations were determined using ϵ_{280} (1%) = 26.4 (Prasad and Litwack, 1963).

Modification of Lysozyme. Lysozyme (2 mg/ml) was reacted with 0.2 *M* **1** in 0.5 *M* sodium bicarbonate buffer (pH 9.0) or 0.1 *M* sodium phosphate buffer (pH 7.0). At various time intervals, an aliquot of this solution was withdrawn and subjected to gel filtration on Sephadex G-25 using 0.1 *M* sodium phosphate buffer (pH 6.0) as the eluent. The uv spectrum of the resulting enzyme was recorded and the modified enzyme was assayed for activity. Hydroxylamine was added to a final concentration of 0.1 *M*, and then after 15 min the protein was extensively dialyzed against water. The uv spectrum of the resulting enzyme was recorded and the activity of the enzyme was measured.

Amino Acid Analysis. Desalted protein samples were hydrolyzed in degassed tubes at 110° for 24 hr in 6 *N* HCl. Hydrolysates were analyzed on a Beckman 120C amino acid analyzer. The analysis of lysine and arginine was accomplished by using the protein's histidine residues as a standard.

Results

Model Studies

Reaction of 2,4-Pentanedione with Primary Amines. The reaction of 2,4-pentanedione (**1**) with *n*-butylamine in benzene leads to the formation of N-*n*-butyl-4-amino-3-penten-2-one (**2**) (Dudek and Holm, 1962). The uv spectrum of enamine **2** (Figure 1) is independent of pH in aqueous solution between pH 6 and pH 12 (λ_{max} 310 nm, ϵ 2.1×10^4 M^{-1} cm^{-1}). In aqueous solution, enamine formation according to eq 1 is reversible, and an excess of amine is required in order to carry the reaction to completion. Rate constants for the reaction of ketone **1** with an excess of *n*-butylamine in aqueous solution at 21° are shown in Table I,

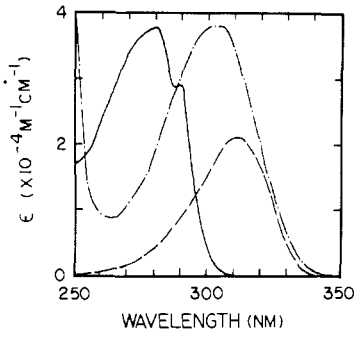
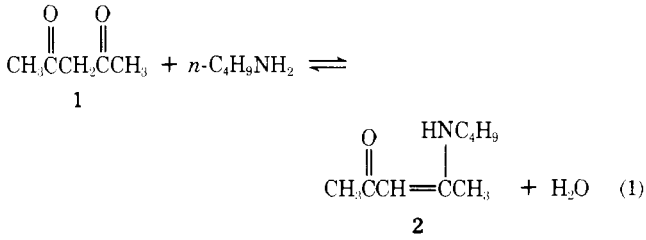
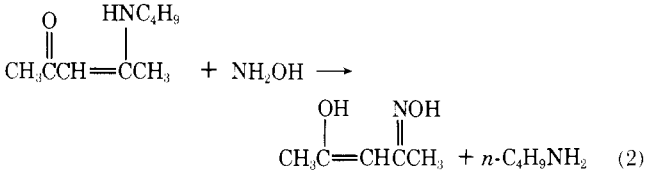


FIGURE 1: Uv spectra of native lysozyme (—); enamine **2** (- - -); and pyrimidine **3c** (-·-·-). The ε of pyrimidine **3c** is multiplied by 10.



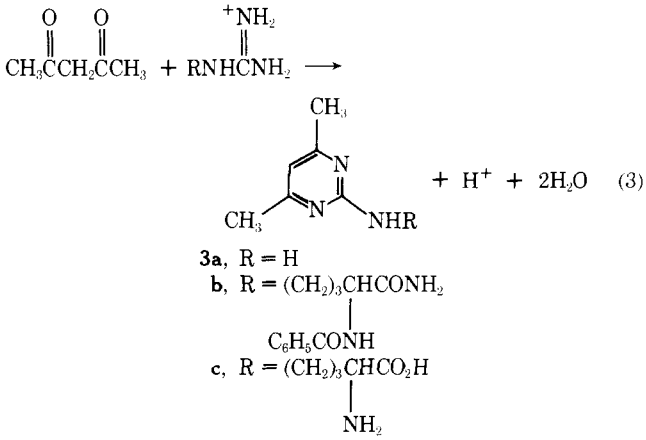
along with rate constants for the hydrolysis of enamine **2** under the same conditions. The rates of enamine formation and hydrolysis are moderately insensitive to pH. Measurements of the rate of enamine formation in the presence of 0.1, 0.2, and 0.3 *M* buffers revealed the presence of buffer catalysis only at pH 7 and below. The pH variation of the equilibrium constants for enamine formation reflects the fact that only unprotonated amine (pK_a = 10.6, Bates and Hetzer, 1961) and neutral **1** (pK_a = 9.0, Izatt et al., 1955) participate in the equilibrium.

Enamine **2** can be destroyed by hydrolysis (eq 1) or more rapidly by reaction with hydroxylamine (eq 2):



The oxime formed lacks significant uv absorbance above 240 nm, so this reaction can conveniently be monitored spectrophotometrically. Bimolecular rate constants for this reaction are given in Table I.

Reaction of 2,4-Pentanedione with Guanidines. Reaction of **1** with an equimolar amount of guanidine, *N*-benzoyl-L-argininamide, or arginine in aqueous solution leads to formation of an *N*-substituted 2-amino-4,6-dimethylpyrimidine as the only product (eq 3). This reaction can easily be monitored spectrophotometrically (**3c** has λ_{max} 300 nm, ε 3.4 × 10^3 M^{-1} cm^{-1}) (Figure 1). Protonation of the 2-amino group (pK_a approximately 5) causes a shift of the λ_{max} to 310 nm and a slight increase in the extinction coefficient. Rate constants for the reaction of **1** with guanidine are shown in Table II. This reaction is more than an order of magnitude slower than reaction of **1** with *n*-butylamine under the same conditions, but the former reaction, unlike the latter, is quite sensitive to buffer concentration and pH.



Aminopyrimidines **3** are stable at 25° between pH 3 and pH 12 for at least several days, and they do not react with hydroxylamine. Hydrolysis of **3b** in 6 *M* HCl at 110° for 24 hr produced quantitative yields of ornithine and 2-hydroxy-4,6-dimethylpyrimidine.

Modification of Lysozyme. Lysozyme (2 mg/ml) was treated with 0.2 *M* **1** at pH 9.0 in 0.5 *M* potassium bicarbonate buffer at 21° for 400 min. After gel filtration the enzyme retained only 20% of its original activity and its uv spectrum (Figure 2) showed increased absorbance above 300 nm due to modification of lysine and arginine. The extent of arginine modification was determined by treatment of a portion of the modified protein with 0.1 *M* NH_2OH for 10 min at pH 6. This treatment increased the activity of the enzyme to 80% of its original value. The enzyme still showed a small absorbance at 310 nm due to the presence of a small amount of modified arginine. Analysis of the uv spectrum of the enzyme by use of the spectra of native lysozyme and **3c** indicated that 0.8 arginine was modified.

Table I: Rate Constants for the Formation, Hydrolysis, and Hydroxylaminolysis of *N*-*n*-Butyl-4-amino-3-penten-2-one (**2**) and Equilibrium Constants for the Formation of the Same Compound as a Function of pH[a] at 21°.

pH	Rate of Formation[b] (M^{-1} min^{-1} × 10^3)	Rate of Hydrolysis (min^{-1} × 10^3)	Equilibrium Constant for Formation[c] (M^{-1})	Rate of Hydroxylaminolysis (M^{-1} min^{-1})
6.0	1.9[d]	4.8	0.4	79
7.0	7.0	1.8	3.9	22
8.0	11.1	0.38	29	3.8
9.0	28	0.20	140[d]	0.48

[a] Measurements were made in 0.2 *M* sodium phosphate buffers (pH 6, 7, and 8) and 0.2 *M* sodium carbonate buffer (pH 9). Experimental values have not been corrected for the ionization of substrates. [b] Calculated from the observed rate of formation of **2** in the presence of 0.2 *M* *n*-butylamine. [c] Determined spectrophotometrically by simultaneous Beer's law analysis for **1** and **2** at equilibrium. [d] Calculated from the ratio of rate constants for formation and hydrolysis of enamine **2**. The concentration of water has not been included.

Table II: Rate Constants for the Reaction of 2,4-Pentanedione with 0.25 *M* Guanidium Sulfate at 21°.[a]

pH	Rate Constant (M^{-1} min^{-1} × 10^4)		
	0.1 *M* Buffer	0.2 *M* Buffer	0.5 *M* Buffer
7.0	1.7	2.6	3.0
8.0	2.9	3.3	4.1
9.0	9.7	14	21
10.0	10	12	22

[a] In sodium phosphate buffer (pH 7 and 8); sodium carbonate buffer (pH 9 and 10).

Table III: Comparison of Spectrophotometric Analysis and Amino Acid Analysis for Measuring the Extent of Modification of Arginine.

Spectrophotometric Analysis	Amino Acid Analysis	
Arginine	Arginine	Lysine + Ornithine
11[a]	11	6
9.3	8.8	6.5
6.5	6.6	9.2
9.4	9.1	5.8

[a] Unmodified enzyme.

Quantitation of the modification of lysine in lysozyme that had been treated with **1** for 400 min could be accomplished by simultaneous Beer's law analysis using the spectra of native lysozyme, **2**, and **3b** (Figure 1), together with the knowledge that this material contains 0.8 modified arginine residue. The spectra were best fitted by assuming that 3.7 of the available six lysines were modified (Figure 2).

The kinetic differential between lysine modification and arginine modification could be increased by conducting the modification reaction at pH 7. Treatment of lysozyme (2 mg/ml) with 0.2 *M* **1** at pH 7 for 24 hr at 21°, followed by the usual chromatographic and spectrophotometric analysis of the extent of lysine and arginine modification revealed that 3.8 lysine residues and less than 0.4 arginine residue were modified. The modified enzyme retained 13% of its original activity, but 95% of the original activity could be recovered by treatment of the modified enzyme with 0.1 *M* NH_2OH at pH 6 for 45 min, followed by gel filtration.

Reaction of lysozyme for 100 hr with 0.2 *M* **1** at pH 9 resulted in the modification of 4 of the 6 available lysine residues and 4.5 of the 11 available arginine residues. Observed and calculated spectra of the modified enzyme after treatment with hydroxylamine are shown in Figure 2. This material showed approximately 15% of the activity of native lysozyme.

The accuracy of the uv analysis for the extent of arginine modification was confirmed by acid hydrolysis and amino acid analysis of the modified protein. The acid hydrolysis converts modified arginine into ornithine, which elutes with the lysine peak in the amino acid analyzer. Quantitation of the arginine modification could thus be accomplished by observing either the increase in the area of the lysine peak or the decrease in the area of the arginine peak with increasing extent of arginine modification. Because of the sizes of the peaks involved, the latter is the more accurate method of analysis. Results of several such analyses are shown in Table III. In general, the results of amino acid analysis agreed with the results of uv analysis within ±0.5 residue. No other differences in amino acid content were observed between native and modified protein.

The possibility of reaction of **1** with other amino acids side chains of proteins was investigated by looking for reactions of **1** with appropriate model compounds. Reaction of **1** with phenol or with mercaptoethanol in aqueous solution pH 9.0 for 48 hr produced no reaction.

Kinetics of Lysozyme Modification. The time course of the modification of lysozyme by 0.2 *M* **1** at pH 9.0, 21°, is shown in Figure 3. Aliquots were withdrawn from the reaction mixture and unreacted **1** was removed by gel filtration. The uv spectrum of the product was used to determine the extent of modification of lysine by the same simultaneous Beer's law procedure as was previously used. At longer reactions times a small correction for arginine modification was included in the calculation. Extent of arginine modification was determined by simultaneous Beer's law analysis following treatment of this modified enzyme with 0.1 *M* NH_2OH and dialysis.

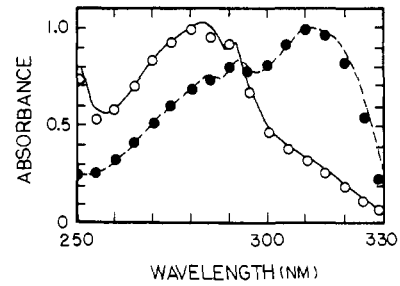


FIGURE 2: Uv spectra of lysozyme treated with 0.2 *M* 2,4-pentanedione for 400 min at pH 9.0 (- - -); calculated points for 3.7 lysine modifications and 0.8 arginine modification (●); lysozyme treated for 100 hr with 0.2 *M* 2,4-pentanedione, then treated with hydroxylamine and dialyzed (—); theoretical points calculated for lysozyme with 4.5 arginines modified (○).

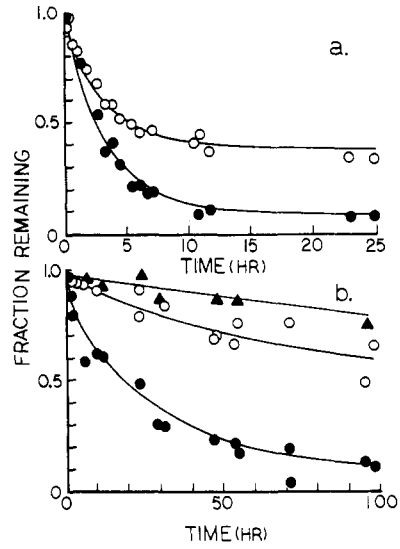


FIGURE 3: Time course of the modification of lysine and arginine in lysozyme with 0.2 *M* 2,4-pentanedione at pH 9.0 and 21°. The ordinate is fraction remaining activity or fraction remaining lysine or arginine. (a) Remaining lysine (○); remaining activity before treatment with hydroxylamine (●). (b) Remaining arginine (○); remaining activity after treatment with hydroxylamine and dialysis (●); activity of control treated exactly as in the modification experiment except for the exclusion of 2,4-pentanedione (▲).

The activity of the modified enzyme was measured both before and after the treatment with NH_2OH. Since lysine modification is much faster than arginine modification, the loss of activity at short reaction times is due almost entirely to lysine modification. Activity loss in the NH_2OH-treated protein is due only to arginine modification.

The modification of lysine under these conditions (with excess **1** present) follows pseudo-first-order kinetics. The second-order rate constant for lysine modification is $2.8 \times 10^{-2}\ M^{-1}\ min^{-1}$, which is the same as the rate constant $2.8 \times 10^{-2}\ M^{-1}\ min^{-1}$ for reaction of *n*-butylamine with **1**. The loss of enzyme activity due to lysine modification proceeds with an identical rate constant of $2.8 \times 10^{-2}\ M^{-1}\ min^{-1}$. Lysine modification stopped when four of the six available lysines were modified, and this modified enzyme

retained 8% of its original activity.

The modification of arginine could only be carried to 50% completion due to the insolubility of the highly modified protein. The modification of the first 4.5 arginines followed pseudo-first-order kinetics, with a second-order rate constant of $1.3 \times 10^{-3}\ M^{-1}\ \text{min}^{-1}$, approximately 20 times smaller than the rate constant for lysine modification under the same conditions, but similar to the rate constant for reaction of **1** with guanidine (Table II). The loss of enzymatic activity due to arginine modification exhibits a second-order rate constant of $2.8 \times 10^{-3}\ M^{-1}\ \text{min}^{-1}$, about twice the rate constant for arginine modification. The activity of the enzyme approaches zero with increasing arginine modification.

Discussion

Reaction of 2,4-pentanedione with primary amines is approximately 20 times faster than reaction with guanidine at pH 9. As a result, reaction of a protein with **1** for a short time leads principally to modification of side chain amino groups of lysine residues. The kinetic advantage of lysine over arginine modification can be further increased by working at pH 7 at low buffer concentration. Modification of arginine is favored by higher pH and higher buffer concentration. Modification of lysine can readily be reversed by treatment with hydroxylamine.

As a reagent for lysine modification, **1** exhibits several useful properties. In contrast to acylating and alkylating reagents, which may react with tyrosine, histidine, cysteine, threonine, serine, or occasionally methionine residues, **1** is specific for lysine modification, except for the much slower arginine modification that occurs at higher pH. The enamine which is formed by reaction of **1** with proteins is relatively stable at pH 8 and above, but the amino group of lysine can be regenerated in a matter of hours by hydrolysis at pH 6 or below or in a matter of seconds by treatment with 0.1 M NH_2OH at pH 6. Measurement of the extent of lysine modification is readily accomplished spectrophotometrically.

As a reagent for the reversible modification of side-chain amino groups of proteins **1** is useful because of the ease with which it can be removed. The only limitation is the instability of the modified amino group below pH 7 and the modification of arginine side chains which occurs at longer reaction times at high pH.

The modification of arginine side chains in proteins with **1** is somewhat slower than the modification with 1,2 dicarbonyl compounds, usually requiring 50–100 hr for extensive reaction. This disadvantage is largely offset by the stability of the product formed on modification of arginine with **1** and by the ease of analysis of the modified product either spectrally or by amino acid analysis. The modification of lysine which occurs under the same conditions is readily reversed by treatment of the protein with hydroxylamine and does not seriously interfere with modification of arginine by this reagent.

Treatment of lysozyme with **1** for 400 min at pH 9 resulted in modification of four out of six available lysine residues and only 0.8 arginine residue. Therefore, the effect of lysine modification on the function of the enzyme could be separated almost completely from the effect of arginine modification. The rate constant for the loss of activity due to lysine modification is the same as that for modification of lysine, assuming that only four of the enzyme's six lysine residues react with this reagent. These results are consistent with those of Davies and Neuberger (1969), who studied the modification of lysine side chains in lysozyme by reaction with acetic anhydride. No evidence for a lysine essential for the catalytic activity of the enzyme was found, but acetylation of lysine residues caused a gradual decrease in catalytic activity attributed to the decrease in electrostatic interaction between the positive charges on the protein and the negatively charged cell wall.

Modification of arginine residues in lysozyme proceeds at a rate that is only one-twentieth of the rate of lysine modification. The rate constant for loss of enzymatic activity associated with modification of arginine is twice the rate constant for modification of arginine, and the enzymatic activity of the modified enzyme approaches zero with increasing degree of modification. It is probable that modification of one arginine residue abolishes the catalytic activity of the enzyme and this modification is approximately twice as fast as modification of the other arginines. Our failure to detect this one rapidly reacting residue can be attributed to the small absorbance change produced by this rapid initial reaction.

The three-dimensional structure of lysozyme (Imoto et al., 1972) has one arginine (Arg-114) at the active site; it is in the substrate binding site at the F site of the cleft. Blocking of this site should eliminate or greatly reduce the catalytic activity of the enzyme. Loss in catalytic activity of lysozyme upon modification of arginine has also been reported by Patthy and Smith (1975) and by Davies and Neuberger (1969). The latter authors found that, as expected, modification of arginine eliminated the activity of lysozyme in cell wall hydrolysis, but did not eliminate the activity of the enzyme toward a tetrasaccharide which does not require the F site for binding.

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Requirement for Protein Synthesis in the Regulation of Protein Breakdown in Cultured Hepatoma Cells†

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ABSTRACT: The modes of action of insulin and of inhibitors of protein synthesis on the degradation of labeled cellular proteins have been studied in cultured hepatoma (HTC) cells. Protein breakdown is accelerated upon the deprivation of serum (normally present in the culture medium), and this enhancement is inhibited by either insulin or cycloheximide. An exception is a limited class of rapidly turning over cellular proteins, the degradation of which is not influenced by insulin or cycloheximide. Alternative hypotheses to explain the relationship of protein synthesis to the regulation of protein breakdown, viz., control by the levels of precursors of protein synthesis, regulation by the state of the ribosome cycle, or requirement for a product of protein synthesis, have been examined. Protein breakdown was not influenced by amino acid deprivation, and measurements of valyl-tRNA levels in HTC cells subjected to various experimental conditions showed no correlation between the levels of charged $tRNA^{Val}$ and the rates of protein degradation. Three different inhibitors of protein synthesis (puromycin, pactamycin, and cycloheximide) suppressed enhanced protein breakdown in a similar fashion. A direct relationship was found between the respective potencies of these drugs to inhibit protein synthesis and to block enhanced protein breakdown. When cycloheximide and insulin were added following a prior incubation of HTC cells in a serum-free medium, protein breakdown was maximally suppressed within 15–30 min. Actinomycin D inhibited protein breakdown only after a time lag of about 90 min. It is suggested that the regulation of protein breakdown in hepatoma cells requires the continuous formation of a product of protein synthesis, in a manner analogous to the mode of the control of this process in bacteria.

In cultured mammalian cells, the rate of degradation of cellular proteins is subject to environmental and hormonal influences. Thus, the withdrawal of serum from the culture medium accelerates the breakdown of labeled cellular proteins in hepatoma cells (Hershko and Tomkins, 1971) and in fibroblasts (Hershko et al., 1971). This "enhanced" protein breakdown is readily slowed down to the "basal" rate upon the supplementation of either serum or insulin (Hershko et al., 1971; Gelehrter and Emanuel, 1974; McIlhinney and Hogan, 1974). Protein breakdown is also enhanced upon exposure of cultured cells to "conditioned" medium (Poole and Wibo, 1973) or following prolonged incubation with dexamethasone (Gelehrter and Emanuel, 1974). Furthermore, the breakdown of the inducible enzyme tyrosine aminotransferase is similarly regulated by the absence or presence of serum (Hershko and Tomkins, 1971).

Some insight into the mechanisms of the regulation of protein breakdown has been provided by the observation that cycloheximide, an inhibitor of protein synthesis, blocks the enhancement of protein breakdown evoked by serum deprivation (Auricchio et al., 1969; Hershko and Tomkins, 1971). On the other hand, the inhibitor has no further influence on the basal rate of protein breakdown that occurs in cells supplemented with serum or insulin. The action of the inhibitor of protein synthesis on enhanced protein breakdown might mean that the stimulation of protein breakdown requires concurrent protein synthesis. Alternatively, it is possible that cessation of protein synthesis causes the accumulation of a precursor (such as aminoacyl-tRNA) which in turn inhibits enhanced protein breakdown (Goldberg, 1971). It may also be that a specific alteration in the state of the protein synthesizing system, produced by the inhibitor, may influence protein breakdown. For example, the state of polysome aggregation (Stanners, 1966) and the availability of various translational factors or of other substances participating in protein synthesis may all be affected by cycloheximide, and these could in some way participate in the regulation of protein breakdown.

Essentially analogous phenomena occur in bacteria where protein degradation is markedly accelerated upon the deprivation of some essential nutrients, such as a nitrogen source, a carbon source, or inorganic phosphate (Mandelstam, 1963; Pine, 1972; Shechter et al., 1973). Here again, inhibitors of protein synthesis prevent the enhancement of protein breakdown elicited by nutritional deprivation (Mandelstam, 1958; Schlessinger and Ben-Hamida, 1966). Our previous studies, utilizing mutant strains of *Escherichia coli* defective in their *rel* loci or containing temperature-sensitive aminoacyl-tRNA synthetases, have led us to the conclusion that in bacteria, the regulation of protein breakdown requires concomitant protein synthesis (Rafaeli-Eshkol and Hershko, 1974; Rafaeli-Eshkol et al., 1974).

Much less information concerning the mechanisms of regulation of protein breakdown is available in animal cells

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