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HYDROXYLATION OF LYSYL RESIDUES IN LYSINE-RICH AND ARGININE-RICH HISTONES BY LYSYL HYDROXYLASE IN VITRO

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Summary

Lysine-rich and arginine-rich histones were examined as substrates for lysyl hydroxylase. Both proteins are known to be rich in lysyl residues, and lysine-rich histone also contains -X-Lys-Gly-sequences, whereas no such sequences are found in the arginine-rich histone. Both histones were found to be hydroxylated by lysyl hydroxylase, and the time courses of the hydroxylation reactions with these substrates were linear for at least 60 min. The K_m values observed where $3 \cdot 10^{-6}$ M for heat-denatured lysine-rich histone and $6 \cdot 10^{-6}$ M for heat-denatured arginine-rich histone.

Heat-denatured lysine-rich histone was hydroxylated at a higher rate than non-denatured both at 37 and 25°C. No such phenomenon was found, however, when arginine-rich histone was examined as a substrate. Furthermore, at 37°C lysine-rich histone was a better substrate for lysyl hydroxylase then arginine-rich histone, but this relationship was reversed at 25°C.

The synthesis of hydroxylysine observed with arginine-rich histone indicates that the lysyl hydroxylase preparation used in these experiments catalyzes the synthesis of hydroxylysine not only in the sequence -X-Lys-Gly-, but also in some other sequences. Certain collagen polypeptide chains are known to contain one hydroxylysyl residue in a sequence other than -X-Lys-Gly-, and the present results may explain this finding.

Introduction

The hydroxylysine and hydroxyproline in collagen are synthesized by hydroxylation of certain lysyl and prolyl residues in a polypeptide precursor of collagen (for reviews, see refs 1—5). These hydroxylations are catalyzed by two separate enzymes, lysyl hydroxylase [6—8] and prolyl hydroxylase [9—11], and both reactions require molecular oxygen, ferrous iron, α-ketoglutarate and a reducing agent, which can be ascorbate [1—5].

The lysyl hydroxylase does not hydroxylate free lysine [12,13] or the tripeptide Lys-Gly-Pro [13], but a single triplet of X-Lys-Gly was found to fulfil a minimum requirement for recognition by the enzyme [13]. The data obtained with various peptides indicated that both the amino acid sequence around the lysine and the length of the peptide chain are important determinants in the synthesis of hydroxylysine by the enzyme [13]. The conformation of the substrate also seems to be a critical factor in the synthesis of hydroxylysine, for it has been demonstrated that the triple-helical structure of collagen and of protocollagen inhibits the hydroxylation of lysyl residues [14,15]. A similar inhibitory effect of triple-helical conformation was also reported in the hydroxylation of prolyl residues by prolyl hydroxylase [16—19].

Lysine-rich and arginine-rich histones are known to be rich in lysyl residues. Lysine-rich histone contains 212 amino acid residues. The amino acid sequence of the first 108 residues from the amino-terminal end was elucidated [20], and was found to contain two triplets of -X-Lys-Gly-. The entire sequence of the arginine-rich histone, which contains 135 amino acid residues, has been determined [20]. There are no sequences of -X-Lys-Gly- in the arginine-rich histones. It seemed to be of interest, therefore, to study whether lysyl residues in these proteins can be hydroxylated by lysyl hydroxylase. Also, the questions of whether there are differences in the synthesis of hydroxylysine between these two proteins, and whether the conformation of these proteins affects the rate of hydroxylysine synthesis were studied.

Materials and Methods

Histones. Lysine-rich and arginine-rich histones from calf thymus were purchased from Sigma (types III and IV, respectively), and their purity was checked by sodium dodecyl sulphate-disc electrophoresis [21]. The lysine-rich histone was found to be over 95% pure, whereas the arginine-rich histone was somewhat more heterogeneous. However, the latter did not contain any detectable band of lysine-rich histone when analyzed by sodium dodecyl sulphate-disc electrophoresis.

Partially purified lysyl hydroxylase. Lysyl hydroxylase was partially purified from the 15 000 × *g* supernatant of 600 g of cartilagenous bones of 15-day-old chick embryos [22] using the same steps as reported for purifying lysyl hydroxylase from the 15 000 × *g* supernatant of whole chick embryos [7]. The preparations were entirely free from prolyl hydroxylase activity, and their specific activities were about 150—400 times that of the 15 000 × *g* supernatant of whole chick embryo homogenates.

Other materials. The peptide L-I was synthesized at the Peptide Center of the Institute for Protein Research, Osaka University, Osaka, Japan. It possessed the amino acid sequence: Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly. Its properties as a substrate for lysyl hydroxylase have been reported previously [13].

α-[1-^{14}C] Ketoglutaric acid was purchased from Calbiochem, and was diluted with authentic α-ketoglutarate (Calbiochem) to give a final specific activity of 60 000 dpm per 0.1 μmol. As reported previously, 30% of the ^{14}C in the preparation of α-[1-^{14}C] ketoglutarate was located in other compounds

[13,23], and the specific activity of the final α-ketoglutarate preparation was corrected accordingly.

Assay procedures. The reaction with lysyl hydroxylase under "standard conditions" was carried out in a final volume of 1.0 ml containing varying amounts of enzyme preparation, 0.05 mM $FeSO_4$, 0.1 mM α-[1-^{14}C] ketoglutarate (60 000 dpm), 0.5 mM ascorbic acid, 0.1 mg of catalase (Calbiochem), 0.1 mM dithiothreitol (Calbiochem), 2 mg bovine serum albumin (Sigma), and 50 mM Tris · HCl buffer adjusted to pH 7.8 at 25°C [7]. The amount of enzyme preparation varied from 35 to 230 μg in different experiments. Unless otherwise noted, the histones and the peptide L-I were heated to 100°C for 10 min and cooled to 0°C just before addition to the incubation system. The samples were incubated at 37 of 25°C for 40 min and the $^{14}CO_2$ was collected onto filter papers as described by Rhoads and Udenfriend [24], except that the reaction was stopped by injecting 1 ml of 1 M potassium phosphate (pH 5.0).

After injection of the phosphate buffer, the sealed tubes were shaken vertically in a mechanical shaker for 30 min, and the filter papers counted in a scintillation mixture containing methyl cellosolve and toluene [25]. All values for the rate of disintegrations per min of the $^{14}CO_2$ released were corrected by reference to the release of $^{14}CO_2$ observed with blank samples that did not contain substrate. In most experiments these blank values varied from 100 to 350 dpm.

All counting of ^{14}C was performed in a Wallac liquid scintillation spectrometer with an efficiency of 85% and background of 25 cpm.

Results

Demonstration of in vitro hydroxylation of lysyl residues in histones

In these experiments lysine-rich and arginine-rich histones were tested as substrates for lysyl hydroxylase. The enzymic reaction was assayed by a method involving the determination of the $^{14}CO_2$ released from the α-[^{14}C] ketoglutarate during the hydroxylation [13]. Previous studies with several different substrates have indicated a stoichiometric relationship between the synthesis of

TABLE I

HYDROXYLATION OF LYSINE-RICH AND ARGININE-RICH HISTONES AT 37°C

Incubation with lysine hydroxylase was carried out at 37°C for 40 min in Expt 1 and for 120 min in Expt 2 as described in Materials and Methods. The substrates were heated at 100°C for 10 min and cooled to 0°C just before incubation with the enzyme. The amount of enzyme used was 50 μg in Expt 1 and 400 μg in Expt 2.

Substrate	Substrate concentration (μg/ml)	$^{14}CO_2$ released (dpm)
Expt 1		
Lysine-rich histone	500	2280
Arginine-rich histone	500	1160
Synthetic peptide L-I	500	5130
Expt 2		
Lysine-rich histone	50	2360
Arginine-rich histone	50	1720

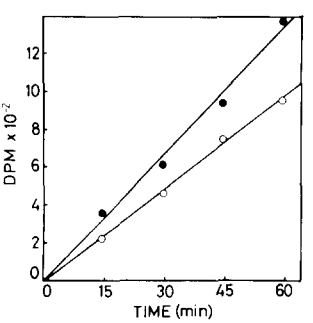
hydroxylysine and the decarboxylation of α-ketoglutarate [13,14]. The substrates were heated at 100°C for 10 min and cooled to 0°C just before the hydroxylation reaction was carried out.

The results indicated that the lysine residues in both lysine-rich and arginine-rich histones were hydroxylated by lysyl hydroxylase in vitro (Table I). When 500 μg of the histones were used as a substrate the release of $^{14}CO_2$ with lysine-rich histone was about twice that observed with arginine-rich histone (Table I, Expt 1).

To obtain maximal hydroxylation of lysyl residues in both histones, small amounts of the histones were incubated with large amounts of the enzyme (Table I, Expt 2). It can be calculated from $^{14}CO_2$ radioactivity released and the specific activity of the α-[^{14}C]ketoglutarate [14] that 3.94 nmol of hydroxylysine were synthesized with the lysine-rich histone, and 2.77 nmol with the arginine-rich histone. The amount of lysine-rich histone used in Expt 2 was 2.28 nmol and that of arginine-rich histone 3.28. Thus, 1.73 nmol of hydroxylysine were synthesized per nmol of lysine-rich histone, and 0.85 nmol per nmol of arginine-rich histone. These results suggest that more than one lysyl residue, possibly two, can be hydroxylated in the lysine-rich histone, whereas possibly one lysyl residue can be hydroxylated in the arginine-rich histone.

The effect of time on the hydroxylation reaction of lysyl residues in histones

The effect of incubation time on the rate of hydroxylation of lysyl residues in lysine-rich and arginine-rich histones was studied by incubating these substrates with lysyl hydroxylase for varying lengths of time. The substrates were denatured at 100°C before the reaction, as described in Materials and Methods, and the incubation was carried out at 37°C. The relationship between time and hydroxylysine synthesis with both substrates was linear for at least 60



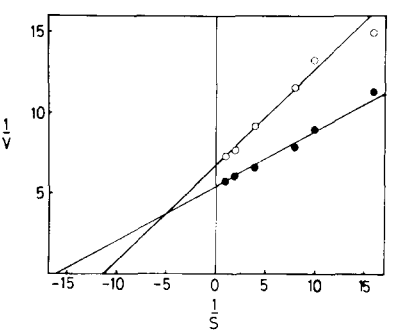


Fig. 1. Time courses for the hydroxylations of lysine-rich histone (●———●) and arginine-rich histone (○———○) at 37°C. The results are the means of two separate experiments. The substrates were heated before incubation as described in Materials and Methods. The enzymic reactions were carried out with 70 μg of lysyl hydroxylase preparation, and the substrate concentrations were 500 μg/ml.

Fig. 2. Double reciprocal plots of initial velocity and substrate concentration in the hydroxylation of lysine-rich (●———●) and arginine-rich histones at 37°C (○———○). Both substrates were heated at 100°C for 10 min, and varying amounts of substrate were incubated with 120 μg of lysyl hydroxylase as described in Materials and Methods. The results are the means of four separate experiments. Abscissa: Reciprocal values of substrate concentration in 10^{-3} μg of substrate. Ordinate: Reciprocal values of the initial velocity in 10^{-4} × dpm of $^{14}CO_2$ released in 40 min.

min (Fig. 1). Lysine-rich histone was hydroxylated at a considerably higher rate than arginine-rich histone.

Determination of K_m for lysine-rich and arginine-rich histones as substrates for lysyl hydroxylase

Double reciprocal plots of initial velocity and substrate concentration for both lysine-rich and arginine-rich histones were determined by incubating varying amounts of these substrates with lysyl hydroxylase for 40 min (Fig. 2). The plots were linear for both substrates. The K_m for lysine-rich histone was 66 μg per ml or $3 \cdot 10^{-6}$ M, and that for arginine-rich histone was 84 μg per ml or $6 \cdot 10^{-6}$ M. The V observed with lysine-rich histone was about 130% of that observed with arginine-rich histone.

Demonstration of the effect of thermal denaturation on the hydroxylation of lysine-rich and arginine-rich histones

Two experiments were carried out to demonstrate the effect of thermal denaturation of the substrate on the hydroxylation of lysyl residues in lysine-rich and arginine-rich histones by lysyl hydroxylase. Incubation with the enzyme was carried out in these experiments either at 37 or at 25°C (Table II). At 37°C, the rate of hydroxylation of lysine-rich histone was found to increase by about 40% if the substrate was denatured prior to incubation with the enzyme. By contrast, heating appeared to have no effect on the rate of hydroxylation of arginine-rich histone. Similar effects were found when incubation with the enzyme was carried out at 25°C. The increase in the rate of hydroxylation of heated lysine-rich histone in these conditions was over 130% of that of non-heated lysine-rich histone. Furthermore, at 25°C, lysine-rich histone was found to be a poorer substrate for lysyl hydroxylase than was arginine-rich histone, although the reverse relationship was found at 37°C (Table II).

TABLE II

THE EFFECT OF HEATING ON THE HYDROXYLATION OF LYSINE-RICH AND ARGININE-RICH HISTONE

The incubations were carried out at 25 and at 35°C for 40 min as described in Materials and Methods. The substrate concentrations in both experiments were 500 μg/l. The amount of the enzyme used in the experiment at 37°C was 50 μg and at 25°C 120 μg.

	Temperature (°C)	Substrate	$^{14}CO_2$ released (dpm)	
			Non heated	Heated*
Expt 1	37	Lysine-rich histone	1600	2280
		Arginine-rich histone	1160	1180
Expt 2	25	Lysine-rich histone	100	230
		Arginine-rich histone	460	460
Expt 3	25	Lysine-rich histone	120	330
		Arginine-rich histone	480	470

* The substrates were heated for 10 min at 100°C and cooled to 0°C just before incubation with the enzyme.

Discussion

Previous studies on the substrate requirements of lysyl hydroxylase indicated that a single triplet of X-Lys-Gly fulfils a minimum requirement for recognition by the enzyme [13], and several natural and synthetic peptides containing such sequences were found to be hydroxylated by lysyl hydroxylase [13—15]. The present data indicating that lysine-rich histone can act as a substrate for the enzyme are in agreement with this minimum requirement, as lysine-rich histone contains at least two such sequences.

However, collagens from bone and embryonic tissues [26—29] are known also to contain one hydroxylysyl residue in the amino-terminal, non-helical portion of the molecule, and this hydroxylysine is found in the sequence -X-Hyl-Ser-. The level of hydroxylation of this residue in bone collagen and in embryonic collagens is generally incomplete [26—29] in that a single tissue contains some collagen molecules in which this position is occupied by a hydroxylysyl residue, and others in which the same position is occupied by a lysyl residue.

Barnes et al. [28] have recently suggested that there may be two lysyl hydroxylases, one of them hydroxylating lysyl residues in the -X-Lys-Gly- sequences, and the other hydroxylating lysyl residues in the -X-Lys-Ser- sequence.

The present results clearly demonstrate that the lysyl hydroxylase preparation which hydroxylated the lysyl residue in the -X-Lys-Gly- sequence of the synthetic peptide L-I also hydroxylated at least one lysyl residue in the arginine-rich histone although the latter does not contain such sequences [20]. This protein contains lysyl residues in the sequences -X-Lys-Ser-, -X-Lys-Thr-, and -X-Lys-Ala [20], the first of these corresponding to the sequence found in the amino-terminal portion of the collagen molecule discussed above. The results thus demonstrate the presence of an enzymic activity in the lysyl hydroxylase preparation from chick embryo cartilagenous bone which can hydroxylate a sequence other than -X-Lys-Gly-. Although these enzyme preparations had been purified up to 400-fold, they were not pure, and it remains to be studied whether a single enzyme hydroxylated all these sequences, or whether the enzyme preparation contained two lysyl hydroxylases with different specificities.

Determination of the K_m values for the two histones as substrates for lysyl hydroxylase indicated that both proteins had lower K_m values than any of the synthetic peptide substrates tested so far. The K_m values for the synthetic peptides have varied from $2 \cdot 10^{-4}$ to $2 \cdot 10^{-3}$ M expressed in terms of the molar concentration of the peptide chain [13]. The corresponding K_m value for the lysine-rich histone was $3 \cdot 10^{-6}$ M and that for the arginine-rich histone $6 \cdot 10^{-6}$ M. The reason for this difference is not known, but previous studies have demonstrated that increasing the length of the peptide chain improves the interaction of substrates with lysyl hydroxylase [13], and thus the low K_m values of the histones compared with those of the synthetic peptides may be related to the considerably higher molecular weights of the former. However, other factors, such as differences in the amino acid sequences [13] may contribute to these differences. On the other hand, it should be noted that

the K_m values for histones are clearly larger than the K_m of about $1 \cdot 10^{-8}$ M which was observed when protocollagen was studied as a substrate for lysyl hydroxylase [15].

The triple-helical structure of collagen has been found to inhibit the enzymic hydroxylation of both lysyl [14,15] and prolyl [16—19] residues in collagen and in protocollagen. It seemed to be of interest to study whether heat denaturation of the histones had any effect on the rate of their hydroxylation. The results indicated a definite effect of heat denaturation on the hydroxylation of the lysine-rich histone, whereas no such effect was found with the arginine-rich histone. The conformations of the histones are not fully understood at the present time. In aqueous solutions they are for the most part in the form of random coils, but neutral salts increase the formation of a helical conformation [20,30]. It has been estimated by the helix prediction method that in the lysine-rich histone there is a helical portion which falls between the residues 47—106, and this prediction has been verified by NMR spectroscopy [30], the results of which have also been interpreted as indicating a β-structure in this region [20]. There are two sequences of -X-Lys-Gly- between residues 47 and 106 in the lysine-rich histone [20], and denaturation of this region may offer an explanation for the effect of heating on the rate of hydroxylation of the lysine-rich histone at 37 and 25°C. The failure to observe any effect of heating on the hydroxylation of the arginine-rich histone suggests either that the hydroxylation occurred in the non-helical portion of the molecule or that the molecule was in a random-coil form. The effect of heating observed with the lysine-rich histone suggests that not only the triple-helical conformation of collagen but also other conformations may interfere with the interaction of lysyl hydroxylase with its polypeptide substrates.

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