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CHARACTERIZATION OF ACTIVATABLE FORM OF PROLYL HYDROXYLASE IN L929 FIBROBLASTS

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Prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate oxygen oxidoreductase, EC 1 14 11 2) activity in a sonicated preparation of early log-phase L929 cells could be increased 3–4-times by preincubation of the sonicate with all cofactors of proline hydroxylation, such as ascorbate, Fe^{2+} and α-ketoglutarate An 'activatable' form of the enzyme is produced in these cells due to a deficiency of one of the cofactors in these cultures The activatable form is found to be different from the active enzyme with respect to its stability to heat and dithiothreitol denaturation. The activatable form has different ionic properties and could be separated from the active enzyme by DEAE-Sephadex chromatography The available evidence suggests that the activatable form is a tight complex produced by the enzyme with underhydroxylated collagen, the latter being produced by a cofactor deficiency in the cells Activation of this complex follows the hydroxylation of the substrate and its subsequent release from the bound enzyme

Introduction

Prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate oxygen oxidoreductase, EC 1 14 11 2) activity in many fibroblasts can be increased by concentrating the cells to a higher density [1,2], or by the addition of sodium lactate [3] or sodium ascorbate [4,5] to the tissue culture medium The fact that this activation is independent of de novo protein synthesis [6] as well as the failure to observe a corresponding increase in prolyl hydroxylase-related antigen [7] can be explained by the conversion of an inactive to an active form

Prolyl hydroxylase is a tetramer and can be converted to monomers by incubation with 10^{-2} M dithiothreitol for 30 min at 30°C [4,8] Addition of ascorbate to dithiothreitol-treated cells increased the activity of the enzyme in cultured cells [4,9] This led to the proposal that prolyl hydroxylase monomers aggregate to form active tetramers in the presence of ascorbate All these studies were done in intact cells

In an earlier report [10], we showed that an activatable form of prolyl hydroxylase could be extracted from L929 fibroblasts and that the activation could be attained in a cell-free system This is done by incubating a sonicated preparation with all cofactors needed for proline hydroxylation, such as ascorbate, Fe^{2+} and α-ketoglutarate, for 3 h at 30°C Since the in vitro activation of prolyl hydroxylase needed all the cofactors of proline hydroxylation, its mechanism should be different from subunit aggregation We had proposed that the activatable enzyme is a tight complex of the enzyme with underhydroxylated collagen and that activation releases the underhydroxylated collagen, thus making the enzyme active In this paper, we give additional evidence to support this hypothesis

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Materials and Methods

α-[1-^{14}C]Ketoglutarate and [3,4-^{3}H]proline were purchased from New England Nuclear (Boston, MA) Sodium lactate was purchased from Fisher Scientific Co (Pittsburgh, PA), sodium ascorbate, α,α′-dipyridyl and L-azetidine-2-carboxylic acid from Calbiochem (La Jolla, Ca) L-Dehydroproline was synthesized by Dr A Felix of Chemical Research Division, Hoffman La Roche (Nutley, NJ) *Cis*-4-Hydroxy-L-proline isolated from sandal leaves was a gift from Dr A N Radhakrishnan Phenazine methosulfate was purchased from Sigma Chemicals (St Louis, MO) and catalase from Boehringer Mannheim (Indianapolis, IN) MPC-11 cells derived from mouse lymphocytes were obtained from S Chen-Kiang and cultured rabbit smooth muscle cells were obtained from J Eichner (in our laboratory)

Culture of L929 cells The conditions for growing L929 cells in monolayer culture were similar to those described by Gribble et al [1], except that ascorbate was omitted from the medium Replicate flasks (Falcon Plastics, 75 cm^2) were incubated with 2 10^6 cells in 20 ml of Eagle's minimal essential medium containing 10% fetal calf serum Most of the activations were carried out with early log-phase cells (40–45 h after inoculation) Cells were harvested with 0 1% trypsin in Dulbecco's phosphate-buffered saline containing 1 mM EDTA and 1 mM glucose and collected by centrifugation The cells were washed twice with the same buffer, then suspended in phosphate-buffered saline (6–8 10^6 cells/ml) and broken by sonication for 15 s at 20 W in a Biosonic II instrument with a needle probe (3 mm diameter)

Suspension cultures of L929 cells were grown as described by Comstock et al [2] Medium without ascorbate was inoculated at an initial cell density of 5 10^4 cells/ml After incubation for 40 h at 37°C with stirring, the cells were harvested by centrifugation, washed and suspended in phosphate-buffered saline (10–30 10^6 cells/ml) and sonicated as above

Activation of prolyl hydroxylase Activation of prolyl hydroxylase in intact cells (in vivo activation) was carried out as described by Stassen et al [4], using either 10^{-5} M ascorbate or 10^{-2} M lactate Activation of prolyl hydroxylase in cell sonicates (in vitro activation) was performed as described earlier [10], by incubating the sonicated cells (0 1 ml) with a mixture containing 0 05 M Tris-HCl (pH 7 6), 1 mM sodium ascorbate, 0 1 mM ferrous ammonium sulfate, 2 mg bovine serum albumin, 400 μg catalase and 0 1 mM α-ketoglutarate in a total volume of 1 0 ml After incubation, 0 1 ml of this mixture was assayed for enzyme activity in a final volume of 1 ml containing additional amounts of each cofactor and tritiated chick embryo substrate The cofactors had to be added again at this point, because those carried over from the in vitro activation were insufficient for the hydroxylation assay Enzyme activity was measured by the ^{3}H-release assay of Hutton et al [11]

Immunologically cross-reacting protein was measured by the enzyme immunoassay of Stassen et al [12], using a monospecific antibody to rat skin prolyl hydroxylase [13] Protein concentration was measured by the method of Lowry et al [14] with bovine serum albumin as the standard

DEAE-Sephadex chromatography Separation of the activatable form of prolyl hydroxylase from active enzyme and smaller cross-reacting antigen was done by DEAE-Sephadex chromatography Early log-phase L929 cells were grown in suspension culture in growth medium containing no ascorbate After harvesting and sonication (25 10^6 cells/ml), a 25 000 × *g* supernatant was treated with 10^{-2} M dithiothreitol for 30 min at 30°C This treatment destroys all the free prolyl hydroxylase but not the activatable form After removing the dithiothreitol by dialysis, the sample was applied to a DEAE-Sephadex column (1 5 × 30 cm) The column was first eluted with 100 ml buffer (0 05 M Tris-HCl (pH 7 4), 0 2 M NaCl, 10^{-5} M EDTA and 10^{-4} M dithiothreitol) Elution was then continued with a linear gradient of 0 2–0 6 M NaCl in the same buffer Aliquots of each fraction (2 6 ml) were assayed for prolyl hydroxylase activity and cross-reacting antigen [12] as well as activatable enzyme

Incorporation of radioactive proline was done by incubating early log-phase L929 cells with 0 25 mCi [3,4-^{3}H]proline/ml Eagle's minimal essential medium containing 10% fetal calf serum for 60 h After incorporation the cells were harvested with trypsin After sonication and centrifugation, a 20 000 × *g* supernatant was treated with 10^{-2} M dithiothreitol to destroy active enzyme After dialysis the sample was applied to a DEAE-Sephadex column as above

The fractions were assayed for prolyl hydroxylase, before and after activation ^3H released into the reaction mixture during activation was measured by distilling (in vacuo) aliquots of the fractions before and after activation and counting the tritiated water

Hydroxyproline determination was done on a pooled, concentrated fraction from the DEAE-Sephadex column containing activatable prolyl hydroxylase Hydroxyproline determination was done before and after activation by acid hydrolysis and reaction with fluorescamine [15]

Prolyl hydroxylase reaction has been shown to involve concomitant release of CO_2 from α-ketoglutarate, which has been shown to be a cosubstrate [16] In order to measure CO_2 liberated during activation, aliquots of a dialyzed preparation from sonicated early log-phase cells were treated with activation mixture in the presence of α-[1-^{14}C]-ketoglutarate The reaction was done in Warburg flasks and $^{14}CO_2$ was trapped in alkali and measured at various intervals of time For each interval, a separate blank which did not contain Fe^{2+} was prepared

Results

In vivo and in vitro activation of prolyl hydroxylase could be seen only in cultures grown in ascorbate-deficient medium [10] and final activities after activation by both methods were the same Neither in vitro nor in vivo activation was seen in cultures from late log-phase cells or purified enzymes from mouse skin and L929 fibroblasts When the cells in the early log-phase were first activated in vivo with ascorbate, the sonicated preparations could not be activated in vitro [10] These experiments suggest that both in vitro and in vivo activation have the same mechanism

Effect of Fe^{2+} deficiency on the formation of the activatable form of prolyl hydroxylase The effect of a deficiency of Fe^{2+} was then studied Fe^{2+} deficiency was induced by addition of an iron chelator, α,α′-dipyridyl, to the medium (Table I) Even though the cells were grown in sufficient ascorbate (0 25 mM, daily addition) [17], the presence of α,α′-dipyridyl produced activatable enzyme in these cells Control cells had higher prolyl hydroxylase activity than those of the treated preparation, and the former did not undergo activation The treated cells, however, had much lower enzyme activity, which increased several-fold upon in vitro activation

TABLE I

EFFECT OF INCUBATION OF L929 CELLS WITH α,α′-DIPYRIDYL ON PROLYL HYDROXYLASE ACTIVITY

Early log-phase L929 cells were grown in the presence of 0 25 mM sodium ascorbate in suspension cultures α,α′-Dipyridyl was added to the cultures and the incubation continued for an additional 18 h The cells were then harvested and assayed for prolyl hydroxylase activity before and after activation with the complete system described in Materials and Methods

	Prolyl hydroxylase (cpm/mg protein)	
	Before activation	After activation
Control	24 190	22 100
α,α′-Dipyridyl (1 mM)	17 700	26 730
α,α′-Dipyridyl (3 mM)	5 100	23 200

Stability of the prolyl hydroxylase present in early log-phase L929 fibroblasts The prolyl hydroxylase activity present in extracts of early log-phase L929 cells is very stable Sonicated preparations of early log-phase cells could be incubated at 37°C for 24 h without loss of prolyl hydroxylase activity and when subjected to in vitro activation, the enzyme activity was increased several-fold In contrast, sonicated preparations of stationary-phase cells rapidly lost their enzyme activity at 37°C and could not be further activated

Another difference in stability between prolyl hydroxylase and the activatable form is demonstrated by the results of dithiothreitol treatment (Table II) Active prolyl hydroxylase has been shown to be a tetramer, which on treatment with dithiothreitol is converted to inactive monomers However, as shown in the table, treatment of a sonicated preparation from early log-phase L929 cells with 10^{-2} M dithiothreitol reduced the amount of enzyme activity present, but did not interfere with the subsequent in vitro activation If the cells were first activated with ascorbate and then treated with dithiothreitol, almost all activity was destroyed, after dialysis, the preparation could not be activated by incubation with

TABLE II

EFFECT OF DITHIOTHREITOL ON PROLYL HYDROXYLASE ACTIVITY IN SONICATES OF EARLY LOG-PHASE L929 FIBROBLASTS

Sonicates were prepared from early log-phase L929 cultures grown in monolayer The 25 000 × *g* supernatants were treated with 10^{-2} M dithiothreitol for 30 min at 30°C After incubation, the dithiothreitol was removed by dialysis against phosphate-buffered saline Prolyl hydroxylase activity was determined in these extracts before and after in vitro activation as described in Materials and Methods

Treatment	Prolyl hydroxylase (cpm/mg protein)	
	Before activation	After activation
No treatment	7 800	21 050
Treatment with dithiothreitol	1 400	14 800
Activation in vivo with ascorbate and treatment with dithiothreitol	3 000	4 220

cofactors The activatable form, therefore, seems to be protected from dithiothreitol denaturation, whereas active enzyme is very labile

Partial purification of the activatable form of prolyl hydroxylase present in L929 cells Earlier separation methods involving gel filtration on Sephadex G-200 and Sepharose 6B did not separate active enzyme from the activatable form However, they did separate active enzyme from smaller cross-reacting protein, and showed that the activatable enzyme has the same or higher molecular weight than the active enzyme, and that smaller cross-reacting protein does not have an immediate role in the ascorbate-induced activation Separation of the activatable form from the active enzyme and smaller cross-reaction protein was achieved by chromatography on DEAE-Sephadex (Fig 1) The activatable form was retarded less than the active enzyme and cross-reacting protein Similarly, it was seen that the activatable form was not bound on an agarose-ascaris collagen affinity column, which does bind the active enzyme [8]

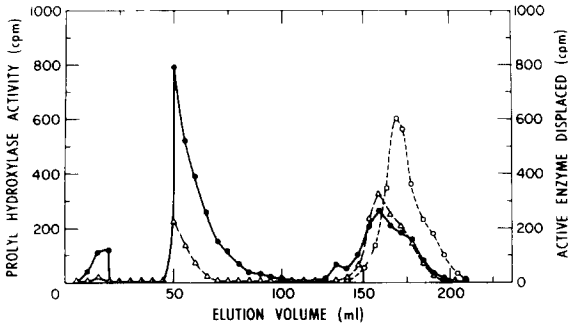

Fig 1 DEAE-Sephadex chromatographic separation of activatable prolyl hydroxylase, active enzyme and cross-reacting protein from early log-phase L929 fibroblasts (△ – – – △) Prolyl hydroxylase activity before activation, (●———●) prolyl hydroxylase activity after activation, (○———○) cross-reacting protein

Mechanism of prolyl hydroxylase activation in L929 cells Available evidence suggests that the activatable form of enzyme is a tight complex with underhydroxylated collagen, which is produced by a cofactor deficiency The following additional evidence is presented to substantiate this hypothesis

Noncompetition with tritiated substrate Deficiency of a cofactor involved in the hydroxylation of proline could result in the formation of underhydroxylated collagen inside the cell This could compete with the radioactive substrate used in the assay, and hence give a lowered value of prolyl hydroxylase However, it was found that the amount of this collagen present in the 10 μl of sonicate used for the assay was so small that it did not interfere in the assay 10 μl of sonicate from early log-phase L929 cells before activation released 347 cpm of ^3H from the substrate during assay 20 μl of purified mouse skin enzyme released 620 cpm of ^3H By mixing these two enzymes, the ^3H released amounted to 1 074 cpm, which is approximately the sum of the two activities If 10 μl of sonicate had enough underhydroxylated collagen which could compete with the radioactive collagen substrate, this value should have been very low, suggesting that the amount of underhydroxylated collagen is very small and that it did not interfere with the assay

^3H release during activation In another experiment, [^3H]proline was incorporated into early log-phase cells, in the absence of ascorbate Under these conditions, proline will be incorporated into underhydroxylated collagen which binds with prolyl hydroxylase DEAE-Sephadex chromatography of this complex is shown in Fig 2 It was found that fractions containing activatable enzyme released ^3H

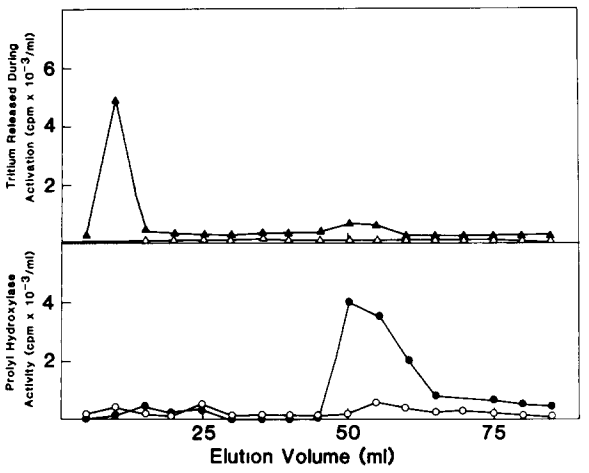

Fig 2 DEAE-Sephadex chromatography of the tritiated activatable form of prolyl hydroxylase from L929 fibroblasts at early log-phase (○———○) Prolyl hydroxylase activity before activation, (●———●) prolyl hydroxylase activity after activation, (△———△) tritiated water in the reaction mixture before activation, (▲———▲) tritiated water in the reaction mixture after activation

into the reaction mixture during activation Since the release of ^{3}H from bound proline is a characteristic reaction during hydroxylation, this gives further evidence that hydroxylation of proline is involved in activation of the enzyme

Analysis of hydroxyproline in DEAE-Sephadex fractions containing activatable enzyme showed that hydroxyproline values doubled after activation

Decarboxylation of α-ketoglutarate during activation Enzymatic hydroxylation of proline is followed by a concomitant decarboxylation of α-ketoglutarate which is a cosubstrate in the reaction In order to see when there is any CO_2 release, activation was performed in the presence of α-[1-^{14}C]ketoglutarate in the absence of any added substrate It was found that activation of prolyl hydroxylase in L929 fibroblast sonicate is followed by a concomitant release of CO_2 (Fig 3), thus showing that substrate hydroxylation is involved during activation

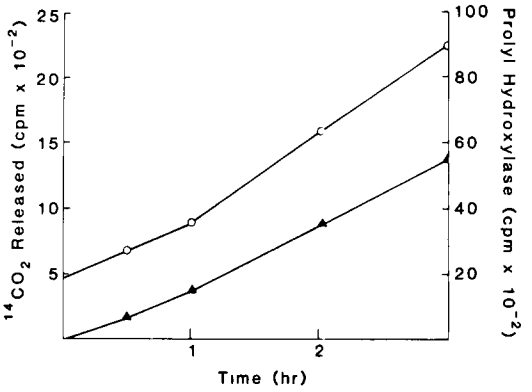

Fig 3 Decarboxylation of α-ketoglutarate during activation (○———○) Time course of prolyl hydroxylase activity during activation, (▲———▲) time course of $^{14}CO_2$ released during activation

Effect of proline analogs on prolyl hydroxylase activity and activatability Previous studies have shown that some of the proline and hydroxyproline residues of collagen can be replaced by the administration of various proline analogs [18,19] Once incorporated into the chain, the analogs cannot be hydroxylated and further collagen synthesis and

TABLE III

THE EFFECT OF PROLINE ANALOGS ON THE ACTIVATION OF PROLYL HYDROXYLASE IN EARLY LOG-PHASE L929 CELLS

L929 cells were grown in replicate flasks for 20 h in the presence of 0 25 mM ascorbate Each analog (1 mM) was added and incubation was continued for another 20 h The cells were harvested and sonicates were assayed for propyl hydroxylase activity before and after in vitro activation

Treatment	Prolyl hydroxylase (cpm/mg protein)		Lactate dehydrogenase (m units/mg protein)
	Before activation	After activation	
Control	23 000	21 880	131
L-Dehydroproline	5 267	3 410	146
L-Azetidine-2-carboxylic acid	9 509	9 303	89
cis-Hydroxy-L-proline	14 500	12 350	138

hydroxylation are inhibited [20,21] Table III shows the effect of these analogs on prolyl hydroxylase activity when they are added to L929 cultures in the early log-phase As can be seen, prolyl hydroxylase activity is decreased and the effect depends on the analog used More importantly for this study, sonicates of cultures treated with the analog could not be further activated in vitro, as analogs could not be hydroxylated Lactate dehydrogenase activity was not affected under these conditions, showing that the analogs had no effect on general protein synthesis

In vitro activation of prolyl hydroxylase in other cell lines Sonicates of 3T3 fibroblasts and a primary cell line derived from rabbit aortic smooth muscle cells could also be activated by incubation with the requisite cofactors (Table IV) In both cell lines it was also possible to increase prolyl hydroxylase activity in cell culture by the addition of sodium ascorbate However, in MPC-11 cells, a line derived from mouse lymphocytes which does not produce any collagen, neither in vivo activation in culture nor in vitro activation of sonicates was observed

Activation of prolyl hydroxylase with phenazine methosulfate In vivo activation of prolyl hydroxylase in early log-phase L929 cells could also be demonstrated using another agent which could produce an oxidation-reduction potential across the cell membrane, such as phenazine methosulfate The optimum concentration to produce maximum activation was found to be 10^{-5} M, and the time required was 3 h The final enzyme activity was found to be the same as that of ascorbate activation (nearly 3-fold) Addition of sodium ascorbate along with phenazine methosulfate did not further increase the prolyl hydroxylase activity in these L929 cells Phenazine methosulfate did not substitute for ascorbate for in vitro activation or in prolyl hydroxylase assay

TABLE IV

IN VITRO ACTIVATION OF PROLYL HYDROXYLASE IN OTHER CELL LINES

All cells were in early log-phase

	Prolyl hydroxylase (cpm/mg protein)	
	Before activation	After activation
3T3	9 000	55 800
Rabbit smooth muscle cells	12 000	54 600
Lymphocytes	4 300	4 300

Discussion

There is considerable similarity between the activation of prolyl hydroxylase in vitro as presented in this paper with that reported in intact cells [4,5] In the intact cells, both α-ketoglutarate and Fe^{2+} are present inside the cell and the only limiting factor is ascorbate, which has been shown to be rapidly decreasing in the tissue culture system [17] When the cells are broken these cofactors are diluted and hence there is a requirement to add them during the in vitro activation Final enzyme activity by both methods was the same When the cells were first activated in vivo, the sonicates could not be further activated in vitro

While cells grown in the absence of ascorbate could be activated in vivo and in vitro, cells grown in the presence of sufficient ascorbate could not be activated by either procedure Similarly, purified prolyl hydroxylase from mouse skin as well as sonicates from stationary-phase L929 cells could not be activated

Addition of iron chelator to cells grown in the presence of sufficient ascorbate produced an activatable form of the enzyme by these cells This shows that the activatable form of the enzyme is produced by the cells in the absence of one of the cofactors of proline hydroxylation

The most likely explanation for the activation phenomenon seen in L929 cells and other cell lines [4,5,22] is that in the absence of ascorbate or Fe^{2+}, cells synthesize underhydroxylated procollagen Several studies have shown that underhydroxylated collagen has a very high affinity for prolyl hydroxylase and forms a stable enzyme-substrate complex [18] This form of the enzyme reacts very slowly with the chick embryo substrate used in prolyl hydroxylase assay When sufficient cofactors are present in vivo or in vitro, underhydroxylated collagen is hydroxylated and dissociated from the enzyme Enzyme is now free to hydroxylate the added chick embryo substrate The events which

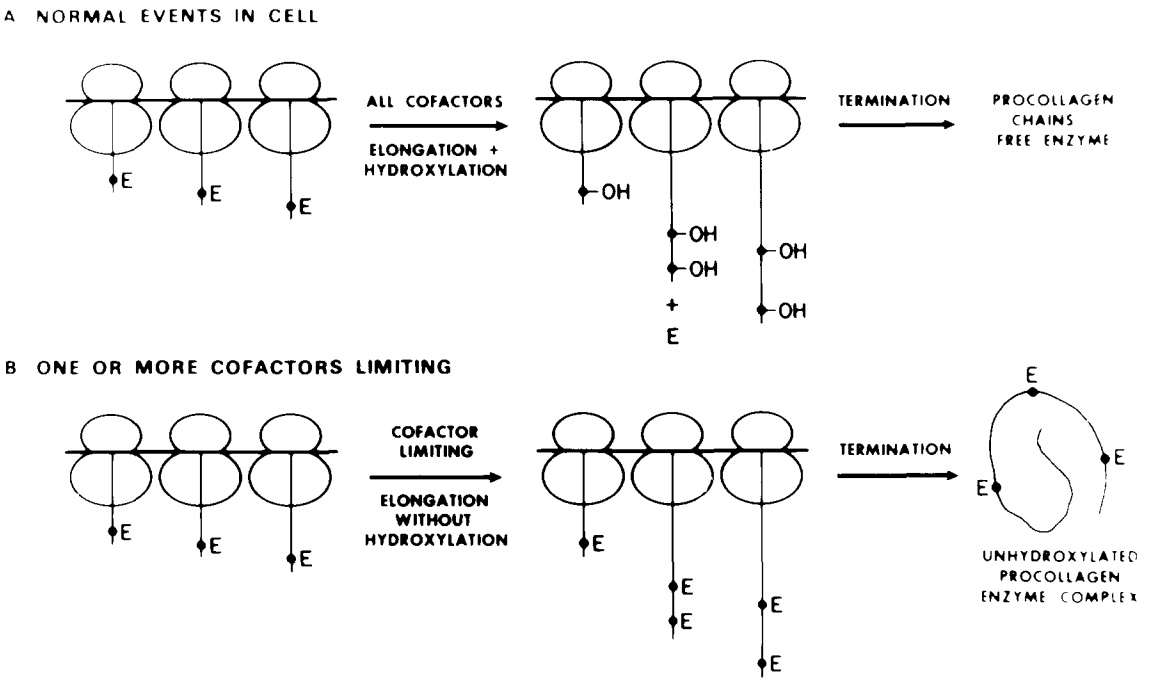

Fig 4 Mechanism of formation of the activatable form of prolyl hydroxylase and it activation in cultured cells The normal events taking place in the presence of sufficient cofactors are depicted in A The enzyme binds to the newly formed collagen while it is still attached to the ribosome As elongation proceeds the molecule is hydroxylated, the affinity for the enzyme greatly decreases and therefore, the enzyme is released In the absence of a reducing agent (ascorbate) or Fe^{2+}, the enzyme still binds to the nascent chains, but now hydroxylation cannot take place and when the collagen is released from the ribosome, the enzyme is still bound to it (B) Therefore, when the cells are sonicated, much of the enzyme is in a form which is inactive until the unhydroxylated collagen can be hydroxylated and removed Since hydroxylation of a native polypeptide chain requires only 15 s, it is reasonable to assume that other underhydroxylated collagen molecules in the sonicate are in equilibrium with the enzyme A minimum hydroxylation of proline residues in these collagen chains is needed for complete activation of the enzyme

appear to take place in the cells with and without sufficient cofactors are shown in Figs 4A and B

In agreement with this hypothesis, we have observed that when a partially purified activatable enzyme (DEAE-Sephadex chromatography) was activated there was an increase in hydroxyproline Secondly, when the cells were labeled with [3,4-^{3}H]-proline in the absence of ascorbate and extracts further chromatographed on a DEAE-Sephadex column, fractions which showed activatable prolyl hydroxylase released ^{3}H into the medium during activation Finally, when the activation was carried out in the presence of α-[1-^{14}C]ketoglutarate (a consubstrate for proline hydroxylase) there was stoichiometric release of $^{14}CO_2$

The possibility exists that the activation of prolyl hydroxylase is due to competition of underhydroxylated collagen present in the cell sonicate with radioactive chick embryo substrate [23] The cumulative evidence shows that a strong enzyme-substrate complex is present in these cells which is resistant to sonication, dialysis, etc Some of this evidence is given below (1) Purified prolyl hydroxylase is not inhibited by the addition of sonicates of early log-phase L929 cells Similarly, addition of increasing amounts of chick embryo substrate does not result in an increased enzyme activity (2) The activatable form of prolyl hydroxylase is more stable to heat denaturation than the active enzyme (3) The activatable form of prolyl hydroxylase is not denatured by the high concentrations of dithiothreitol whereas active enzyme is denatured (4) It has been shown earlier that activation of prolyl hydroxylase could be demonstrated using a different

assay [22] which employs an excess of substrate

The inability to activate prolyl hydroxylase in late log- or stationary-phase cells is not unexpected It has been shown that even if ascorbate is not added to such cultures they are capable of synthesizing hydroxyproline [24] Therefore, all prerequisite cofactors must be present in stationary-phase cells and the collagen formed will be fully hydroxylated and not capable of binding with the enzyme The nature of the reducing agent which serves as a cofactor in these cells is not clearly known [25]

Even though chromatographic attempts at characterizing the activatable form of prolyl hydroxylase were not completely successful, they did show that it was possible to obtain a partially purified preparation which could be activated in vitro This material was as large or larger than the enzyme [10] but had different ionic properties The smaller enzymatically inactive, but immunologically cross-reacting, protein which is a monomer of prolyl hydroxylase does not have an immediate role in the ascorbate-mediated prolyl hydroxylase activation Using radioactive labelling techniques this cross-reacting protein has been definitely known to be a precursor of prolyl hydroxylase [9,26,27] Activation of this antigen to active enzyme follows a mechanism not known at the present time

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