AN ACTIVATABLE FORM OF PROLYL HYDROXYLASE IN FIBROBLAST EXTRACTS

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SUMMARY: An *in vitro* increase in prolyl hydroxylase activity has been effected in sonicates of early log phase L 929 mouse skin fibroblasts from either monolayer or suspension cultures. The requirements for activation are identical to those needed for the hydroxylation reaction itself, i.e., ferrous ion, ascorbate and α-ketoglutarate. Catalase, which is not an absolute requirement for the hydroxylation, is also necessary for activation. The activation is time dependent and, under the conditions used, is complete in 3 hr at 30°. Since ferrous ion also appears necessary for the activation in intact cells and since the same level of activation is achieved in intact cells as in sonicates, it appears that the *in vitro* activation proceeds in the same manner as that seen in cultured cells.

Fibroblast cells in culture produce a significant amount of hydroxyproline only after the cells reach late log-phase (1). Gribble *et al.* (2) showed that increased hydroxyproline formation in these cells is accompanied by a sharp increase in the activity of prolyl hydroxylase (EC 1.14.11.2). An increase in the activity of prolyl hydroxylase in these cells in early log-phase can be induced by concentrating the cells to a higher density (3) or by the administration of sodium lactate (4) or sodium ascorbate (5,6). It was subsequently shown that the increased activity in cellular prolyl hydroxylase was not due to formation of new enzyme but to the activation of an already existing protein which cross-reacts with specific antibody to the enzyme (7). Since most of the immunologically cross-reacting protein was of a much lower molecular weight than active enzyme, we originally considered that enzyme activation was a result of some type of subunit aggregation (5,8). However, all former studies were based on activation of prolyl hydroxylase in intact cells. Recently we have been able to prepare ex-

tracts of fibroblasts which permit activation in vitro. These studies reveal that a mechanism other than subunit aggregation is responsible for this enzyme activation.

MATERIALS AND METHODS

Sodium lactate was purchased from Fisher Scientific Company, sodium ascorbate and α,α' dipyridyl from Calbiochem and catalase from Boehringer Mannheim.

Cells were grown in suspension culture as described earlier (3). Medium was inoculated with an initial cell density of 40,000-50,000 cells/ml. After incubation for 40 hr at 37° in the presence of 95% O_2 and 5% CO_2, the cells were harvested and washed with Dulbecco's phosphate buffered saline. Monolayer cells were grown as described earlier (4) and harvested with trypsin. Cells from both types of culture were resuspended in the same buffer (10-30 million cells/ml) containing dithiothreitol (10^{-4} M) and were broken by sonication for 30 sec at 20W on a Biosonik II instrument equipped with a needle probe 3 mm in diameter.

Activation of prolyl hydroxylase in intact cells (in vivo activation) was carried out as described by Stassen et al. (5). Activation of prolyl hydroxylase in cell sonicates (in vitro activation) was performed by incubating the cell sonicate (0.1 ml) with a mixture containing Tris.HCl (0.05 M, pH 7.6), ascorbic acid (1.0 mM), ferrous ammonium sulfate (0.1 mM), bovine serum albumin (2 mg), catalase (400 μg) and α-ketoglutarate (0.1 mM) in a total volume of 1 ml. The solution was incubated for 3 hr at 30° with shaking. After the incubation period, 0.1 ml of this mixture was taken for enzyme assay. This was diluted to a final volume of 1.0 ml in the presence of an additional amount of each cofactor (9) and tritiated chick embryo substrate (10). Cofactors were required at this point because those carried over from the in vitro activation were insufficient for the hydroxylation assay. Enzyme activity was measured by the tritium release assay of Hutton et al. (10) as described by McGee et al. (7).

Immunologically cross-reacting protein was measured by the enzyme immunoassay of Stassen et al. (11) using a monospecific antibody to rat skin prolyl

hydroxylase. Protein concentration was measured by the method of Lowry et al. (12) with bovine serum albumin as standard.

RESULTS

When sonicates of early log-phase L 929 cells grown in suspension culture were incubated for 3 hr with the cofactors required for proline hydroxylation, and then assayed for prolyl hydroxylase activity, it was observed that the enzyme activity in the sonicates was increased several fold (Table I). All the

TABLE I

REQUIREMENTS FOR THE ACTIVATION OF PROLYL HYDROXYLASE IN SONICATES OF EARLY LOG-PHASE L 929 FIBROBLASTS FROM SUSPENSION CULTURE

Additions to Sonicate for Activation	Prolyl Hydroxylase Activity %
None	100
Complete system	744
Complete system (kept at 0°)	173
Complete system - Ascorbate	105
Complete system - Fe^{++}	303
Complete system - α-Ketoglutarate	274
Complete system - Catalase	107
Complete system - Bovine serum albumin	750

A sonicate from early log-phase cells grown in suspension culture was dialyzed against Dulbecco's phosphate buffered saline to remove free cofactors. An aliquot (0.1 ml) of this sonicate was treated with cofactors and incubated for 3 hr at 30°. The complete system contained Tris.HCl (0.05 M, pH 7.6), ascorbate (1.0 mM), Fe^{++} (0.1 mM), bovine serum albumin (2 mg), catalase (400 µg) and α-ketoglutarate (0.1 mM) in a total volume of 1.0 ml. Following the incubation, 0.1 ml of this mixture was assayed for prolyl hydroxylase activity after further addition of the required cofactors (7) and tritiated chick embryo substrate (10) in a final volume of 1 ml.

cofactors, i.e., ascorbic acid, ferrous ion and α-ketoglutarate, which are needed for the hydroxylation of proline were also needed for the activation. α-Ketoglutarate could not be replaced by oxaloacetate or pyruvate. There was also an absolute requirement for catalase which could not be replaced by heat-denatured catalase. This requirement for catalase is probably due to hydrogen peroxide production during the long period of incubation necessary for maximal activation. An oxygen requirement could not be shown for the activation. However, with the anaerobic system used, the hydroxylation reaction itself was inhibited only 50%. Therefore, if the activation requires oxygen, enough could have been present in the system used in these studies.

The activation was time dependent and under the conditions described above, activation was complete in 3 hr at 30°. Incubation at 37° did not further increase enzyme activity nor did using higher concentrations of cofactors. The time course of activation of prolyl hydroxylase is shown in Fig. 1. In vivo activation with sodium ascorbate in L 929 cells showed a similar time course (5).

There are a number of other indications that the in vitro activation described here is the same as the in vivo activation in tissue culture described

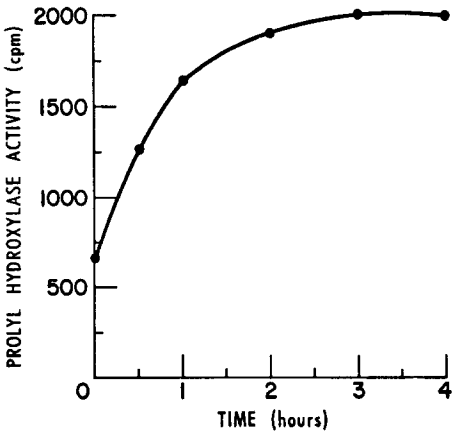

Fig. 1 Time course of in vitro activation of prolyl hydroxylase. Details are given under Materials and Methods.

earlier. The amount of enzyme activity after activation by both *in vivo* and *in vitro* methods was found to be the same. When monolayer cells were first activated *in vivo* with sodium ascorbate there was little further activation of the sonicates *in vitro* (Table II). Also, when the cells were grown with sufficient ascorbic acid (13), it was found that both the *in vivo* and *in vitro* activation was reduced to the same extent.

Further evidence that the *in vitro* and *in vivo* activation are identical was obtained when it was shown that the *in vivo* activation also requires ferrous ion. In the presence of α,α' dipyridyl (3.0 mM), the prolyl hydroxylase activity could not be increased *in vivo* by the addition of ascorbate or lactate.

In order to characterize the activatable enzyme, a 20,000 x g supernatant from early log-phase L 929 cells was chromatographed on a Sepharose 6B column (1.6 x 95 cm) and eluted with Veronal buffer (0.01 M, pH 7.0) containing 0.1 M NaCl (Fig. 2). Fractions (1.2 ml) were assayed for prolyl hydroxylase activity (Fig. 2B) and for protein antigenically related to the enzyme (Fig. 2A) before and after activation with cofactors. Before activation, antigen was present in

TABLE II

COMPARISON OF *IN VIVO* AND *IN VITRO* ACTIVATION FOR PROLYL HYDROXYLASE IN EARLY LOG-PHASE L 929 MONOLAYERS

Treatment	Prolyl Hydroxylase Activity (CPM/mg protein)
None	5,800
Activation with ascorbate *in vivo*	21,800
In vitro activation of the sonicate with cofactors	19,700
In vivo activation with ascorbate followed by *in vitro* activation	24,800

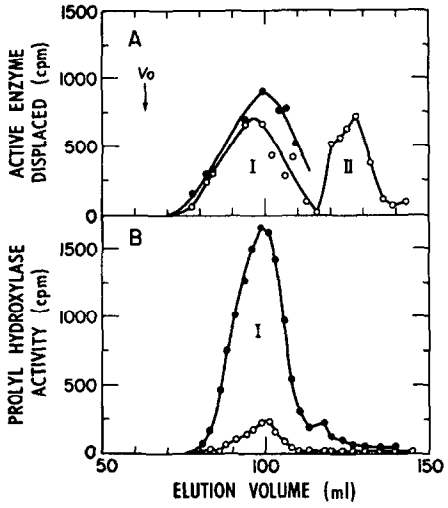

Fig. 2 Sepharose 6B chromatography of a supernatant of early log-phase L 929 cells grown in suspension culture. The column (1.6 x 95 cm) was eluted with 0.01 M Veronal buffer, pH 7.0 containing 0.1 M NaCl. Frame A depicts antigen as measured by the enzyme immunoassay (11) on the fractions before (0) and after (●) activation with cofactors. Peak II corresponds to the low molecular weight cross-reacting protein described previously (7). Frame B shows enzyme activity before (0) and after (●) activation.

peaks I and II but enzyme activity was found only in peak I. After activation, the amount of antigen in peak I increased slightly, but the enzyme activity increased tenfold. However, peak II which corresponds to the low molecular weight cross-reacting protein referred to previously (7,8) still exhibited no enzyme activity. This chromatographic procedure does not separate enzyme from activatable enzyme. However, it does demonstrate that the activatable form of prolyl hydroxylase is as large or larger than active enzyme and that the smaller cross-reacting protein (7,8) is not converted to active enzyme by incubation with the cofactors.

DISCUSSION

The results presented in this paper indicate that the same type of activation of prolyl hydroxylase which previously could only be carried out in whole cells

can now be effected in sonicates. The fact that cells which had been activated in culture could not be further activated *in vitro* points to a similar mechanism for both the *in vivo* and *in vitro* activation. This is also supported by the findings that the *in vivo* and *in vitro* activation resulted in the same level of enzyme activity and by the apparent requirement for ferrous ion in the *in vivo* system as well as in the *in vitro* system.

Preliminary evidence indicates that in addition to active enzyme and the smaller cross-reacting protein, these cells may have a third enzyme-related protein which is capable of being activated. The activatable enzyme was found to be thermally more stable than the enzyme. Treatment with dithiothreitol (0.01 M), which dissociates the enzyme into inactive subunits, did not have any effect on the activatable enzyme. Moreover, separation of activatable enzyme, active enzyme and cross-reacting protein was achieved by DEAE-Sephadex chromatography; details of which will be published elsewhere. Neither the active enzyme nor the smaller cross-reacting protein could be further activated *in vitro*.

Since the *in vitro* activation requires all of the cofactors necessary for hydroxylation, the possibility exists that activation is dependent on hydroxyproline formation. If this is the case, then the activatable enzyme may be some form of complex between active enzyme and an unhydroxylated form of collagen. As long as this complex exists the enzyme cannot react with exogenous substrate and its activity would appear to be very low. Such a hypothesis is consistent with the finding that by limiting prolyl hydroxylase activity by treatment with α,α' dipyridyl, incubation in a nitrogen atmosphere or ascorbic acid deficiency, unhydroxylated collagen accumulates in the cell and is not secreted (14,15). Berg and Prockop recently showed that such unhydroxylated collagen has a high affinity towards prolyl hydroxylase ($K_m \sim 2$ nM) (16). If the activatable enzyme were such a complex then in the presence of the required cofactors and oxygen the bound collagen would be hydroxylated and its affinity reduced so as to free the enzyme for reaction with added substrate. In this way hydroxylation could account for the activation. Consistent with this interpretation is the initial

finding that in vitro activation of a partially purified preparation of the activatable material results in an increase in its hydroxyproline content. Previous studies have shown that pure prolyl hydroxylase contains no hydroxyproline (17,18).

Similar in vitro activation has now been demonstrated in 3T3 fibroblasts and in a primary cell line isolated from rabbit aortic smooth muscle tissue. It appears likely that the in vivo activation observed in these cells (6) occurs by a similar mechanism.

Although these results indicate that the smaller immunologically cross-reacting protein is not activated by this technique they do not lessen the interest in this widely distributed (11) cross-reacting protein. Its significance still merits investigation.

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