STIMULATION OF PROTOCOLLAGEN PROLINE HYDROXYLASE ACTIVITY BY NUCLEOSIDE TRIPHOSPHATES

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SUMMARY

Activity of purified protocollagen proline hydroxylase was enhanced several fold by addition of nucleoside triphosphates (3 mM) to the assay medium, but nucleoside mono- and diphosphates were almost inactive. Pyrimidine nucleotides were less effective compared with purine nucleotides, among which GTP was the most effective. dATP and ATP analogues such as adenosine 5'-(β,γ-imino) triphosphate (AMP-PNP), adenosine 5'-(β,γ-methylene) triphosphate (AMP-PCP), etc. were inactive. ATP or GTP showed no additive effect on enzyme activity stimulated by dithiothreitol or bovine serum albumin.

Protocollagen proline hydroxylase [proline, 2-oxoglutarate dioxygenase: EC 1. 14. 11. 2], one of the new and unusual class of mixed function oxygenases, shows a stoichiometric decarboxylation of α-ketoglutarate accompanied with the proline hydroxylation (1), requires α-ketoglutarate (2), ferrous ion (3,4), ascorbate (3,5) and the atmospheric oxygen (6). As purification of the enzyme progressed some additional factors such as serum albumin, dithiothreitol (7,8,9) and catalase (10), were found to stimulate enzymatic activities, even in the presence of cofactors.

With reference to the regulation of this enzymatic activity in vivo, there has been little information except some reports obtained from studies on the enzyme of cultured fibroblasts. Thus, the activity of the protocollagen proline hydroxylase in cultured early-log phase cells was found to increase by several-fold if lactate (11), ascorbate (12), or the isolated factor from experimentally injured liver (13) was added to the medium. It was presumed that ascorbate or lactate may bring about a conversion of the

Abbreviations: AMP-PNP, adenosine 5'-(β,γ-imino) triphosphate; AMP-PCP, adenosine 5'-(β,γ-methylene) triphosphate; AMP-CPP, adenosine 5'-(α,β-methylene) triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol

inactive precursor to the active enzyme (14). However, the effect could not be a direct one since the cells had to be intact to produce the observable stimulation of enzymatic activity. In the course of studies on the regulation of protocollagen proline hydroxylase activity, we observed that the activity of the enzyme was considerably enhanced by the addition of ATP to the reaction mixture. In this communication we report the stimulation in vitro of protocollagen proline hydroxylase by various nucleotides.

MATERIALS AND METHODS

Adenosine 5'-(β,γ-imino) triphosphate (AMP-PNP) and adenosine 5'-(β,γ-methylene) triphosphate (AMP-PCP) were obtained from Boehringer Mannheim Corp., adenosine 5'-(α,β-methylene) triphosphate (AMP-CPP) was purchased from Miles Laboratories. [4-^{3}H] proline (21 Ci/mole) was a product of Schwarz Bio Research, Inc..

Assay of Enzyme Activity: Protocollagen proline hydroxylase activity was assayed essentially according to the method of Hutton, et al (15). Each assay mixture had a volume of 1 ml and contained the following; Tris-HCl buffer, pH 7,5, 200 μ moles; $FeSO_4$, 2 μ moles; sodium ascorbate 10 μ moles; α-ketoglutarate, 2 μ moles; catalase, 200 μg; peptidyl [4-^{3}H]-substrate (60,000 cpm); enzyme preperation (0.1-1.0 μg of protein). The substrate was prepared by incubating minced rat fetuses with [4-^{3}H] proline in the presence of α,α'-dipyridyl, followed by extracting the labelled protocollagen according to the same authors.

The reaction was carried out for 15 min at 37° with shaking in air and stopped by adding 0.2 ml of 50 % trichloroacetic acid. Tritiated water formed was collected by vacuum distillation. Radioactivity was determined in a liquid scintillation spectrometer using Bray's solution.

Preperation of Enzyme: The enzyme was purified from decapitated 12-day-old chick embryos according to the method of Rhoads and Udenfriend (9), using ammonium sulfate fractionation (30-65 %), DEAE-Sephadex chromatography, ammonium sulfate extraction, and passage through two Sephadex G-200 columns.

Table I

Effect of various nucleotides on the protocollagen proline hydroxylase activity

Additives	^{3}H-water formed(cpm)	% of control
None (Control)	483 ± 16	100
AMP	355 ± 11	73
Cyclic-AMP	432 ± 7	89
GMP	418 ± 10	87
ADP	522 ± 32	108
GDP	418 ± 10	149
ATP	1503 ± 24	311
GTP	2644 ± 13	486
ITP	1995 ± 22	365
CTP	1160 ± 13	212
UTP	1049 ± 10	192

The standard incubation conditions are given in Materials and Methods. Concentration of the nucleotides used was 3 mM. Values are means ± S.E. of four determinations.

The peak of enzymatic activity eluted from Sephadex column was coincident with the small peak of protein eluted in the same position, and the specific activity was 2,000-fold greater than that of the original 105,000g supernatant fraction.

RESULTS AND DISCUSSION

As shown in Table 1, addition of ATP resulted an enhancement of protocollagen proline hydroxylase activity by 2-4-fold. This stimulatory effect was even seen at the nucleotide concentration as low as 0.1 mM, and the maximum stimulation was attained at 3 mM ATP. Nucleoside triphosphates other than ATP were also effective in the stimulation; GTP was more effective than ATP, and it enhanced the enzymatic activity by about 5-fold. Occasionally the enhancing effect of GTP reached upto 8-fold of the control (Figure 1). Among the nucleoside triphosphates, pyrimidine nucleotides, CTP

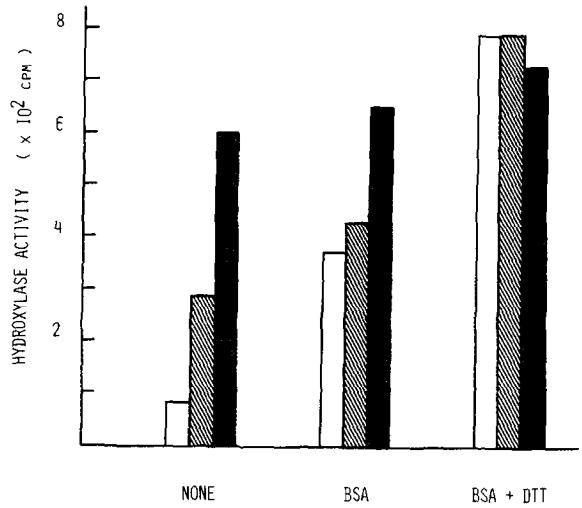

Figure 1. Effect of BSA and DTT on the nucleotides-stimulation of protocollagen proline hydroxylase. The standard incubation conditions are given in Materials and Methods. Left (shown as NONE); Neither BSA nor DTT, Middle; BSA (2 mg/ml) solely, Right; Both BSA (2 mg/ml) and DTT (0.01 mM), were added, respectively. ▭ , no nucleotide; ▨ , ATP (3 mM); ▩ , GTP (3 mM).

and UTP showed less effect compared with purine nucleotides. Nucleoside mono- and diphosphates were inactive. In addition, ADP inhibited the stimulatory effect of ATP competitively and that of GTP to a lesser extent. On the other hand, GDP was a more potent inhibitor of stimulatory activities of both ATP and GTP. These results suggest that the affinity of guanine nucleotides is stronger than that of adenine nucleotides.

AMP-PNP, AMP-PCP and AMP-CPP, analogues of ATP (16), were all ineffective in enhancing the enzymatic activity, and some of them were inhibitory. dATP was also ineffective. These data may suggest the possibility that the stimulatory effect of nucleoside triphosphates would be energy-dependent, but it is not yet clear that the breakdown of γ-phosphate is really necessary in this activation process.

Lineweaver-Burk plots of the data showed that the control and nucleotides-stimulated activities exhibited a little difference in the affinity of enzyme for substrate, and that the primary action of nucleotides was to increase Vmax.

As shown in Fig. 1, addition of BSA (2 mg) to the assay medium resulted the enhancement of the enzymatic activity by 4.6-fold, in agreement with the report of Rhoads et al. (9). In the presence of BSA, however, GTP and ATP were less active, showing stimulations of only 1.1- and 1.5-fold, respectively. Similarly, dithiothreitol (DTT) at 0.1 mM concentration enhanced the enzymatic activity markedly, and in this case, the nucleotides did not stimulate it any more (not shown). Although 0.01 mM DTT showed no stimulation, BSA (2 mg) together with it showed more enhancing activity than BSA alone, i.e., by 10-fold. Also in the presence of BSA and DTT at these concentrations, neither GTP nor ATP caused further enhancement of the enzymatic activity. DTT above 0.1 mM concentration was rather inhibitory, and the activity was not restored by addition of GTP or ATP.

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