THE OCCURRENCE OF A PROTEIN INHIBITOR FOR 5'-NUCLEOTIDASE IN EXTRACTS OF ESCHERICHIA COLI

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Received July 15, 1966

Protein inhibitors have been discovered for a number of bacterial enzymes, including NADase (1), NAD pyrophosphatase (2), UDPGase (3) and ADPGase (3), and it has been suggested that such inhibitors may play a regulatory role in cell metabolism (4). In the course of a survey of enzymes released by osmotic shock (5) we noted the presence of UDPGase in the shock fluid although no enzyme activity could be detected in extracts of normal cells. Furthermore, the UDPGase activity of shock fluids could be neutralized by mixing with dialyzed cell extracts. At this point Dr. Glaser informed us of similar work going on in his laboratory, the results of which had just been submitted as a preliminary note (3). Our results were in general agreement, and we thereupon decided to investigate whether protein inhibitors might be found for other enzymes released by the shock procedure. We were especially interested in 5'-nucleotidase because the low activity usually found in cell extracts could be increased by incubation at 37° after dilution with water (6). However, in this earlier work no clear-cut evidence for an inhibitor was obtained. This was due to the fact that the prescribed conditions for enzyme assay (6) led to partial reactivation of the inhibited enzyme. In the present study assays were run at 22°, and the 5'-nucleotidase of E. coli extracts was found to be almost completely inhibited. Furthermore, the presence of excess inhibitor could be demonstrated by the ability of such extracts to neutralize added free enzyme.

Materials and Methods--E. coli K37 were grown to stationary phase in a semi-

complex medium high in phosphate (Medium B), and subjected to osmotic shock exactly as described in a recent paper (6). Cells harvested in mid-exponential

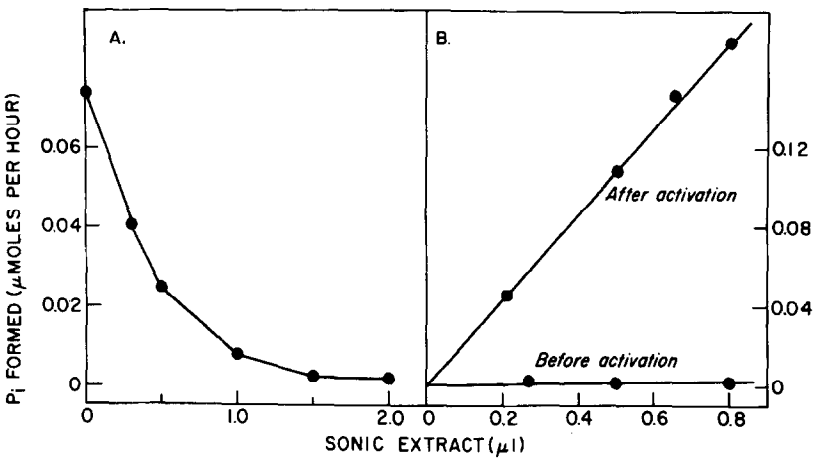

Fig. 1. Evidence for an inhibitor of 5'-nucleotidase in extracts of E. coli. (A). An extract of shocked cells (5.7 mg protein per ml), in the amounts shown, was incubated at 22° for 30 min. with 3 μl. of shock fluid (0.4 μg protein). The volume (0.03 ml) was then increased to 0.1 ml with addition of 5'-AMP and salts (6), and formation of P_i was measured after 20 min. at 22°. No significant 5'-nucleotidase could be measured in the extract itself under these conditions. (B). An extract of intact cells (6 mg protein per ml) was assayed at 22°, with or without prior activation. Activation consisted in diluting the extract 140-fold with water and keeping it at 37° for 60 minutes.

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phase were also examined. Sonic, French press and alumina extracts of washed cells were centrifuged at 100,000 x g for 1 hour. The assay for 5'-nucleotidase has been described (6). For UDPGase the reaction mixture (0.1 ml) contained 0.1 M Tris, pH 7.4, 0.01 M $MgCl_2$, 1.4×10^{-3} M UDPG and excess purified E. coli alkaline phosphatase. Liberation of P_i, and occasionally of glucose (3), was followed.

Results and Discussion--Fresh extracts showed extremely low 5'-nucleotidase activity when assayed at 22°, amounting to 1% or less of that obtained after prior heating of a greatly diluted extract for 1 hour at 37° (Fig. 1, B). When increasing amounts of extract were mixed with shock fluid containing free enzyme, there was progressive inactivation (Fig. 1, A). The results of assays conducted at 37° were complicated because substantial activity was measured

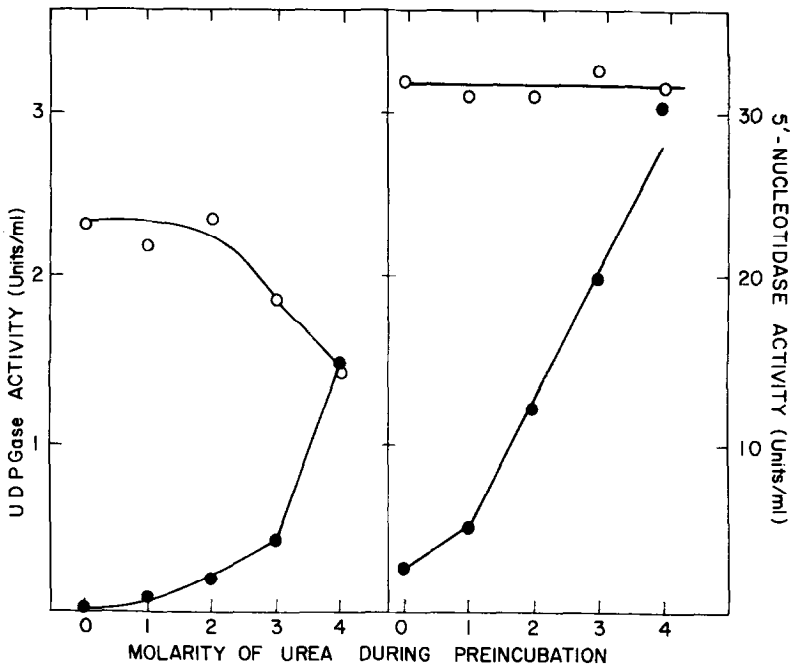

Fig. 2. Activation of cell extracts by urea. A centrifuged sonic extract (1.5 mg protein per ml) was treated at 22° for 20 min. with the indicated concentrations of urea. Closed circles indicate 5'-nucleotidase (at 22°) or UDPGase (at 37°) measured after 10-fold dilution in the assay mixtures. Open circles show effect of same treatment on free enzymes present in shock fluid (0.11 mg protein per ml). Activities are expressed as μmoles substrate hydrolyzed per hour per ml of enzyme solution.

even in the presence of a large excess of inhibitor. Presumably this represents destruction of inhibitor in the course of the assay.* The inhibited 5'-nucleotidase activity of extracts could also be released by heating at 58° for 5 minutes, as described for UDPGase by Melo and Glaser (3). Here again, the extent of activation was greater in quite dilute solutions (20 μg protein per ml). Treatment with 4 M urea was a third method for activating the two enzymes (Fig. 2).**

* By contrast, no significant UDPGase activity was detected in fresh extracts at either temperature. The free UDPGase contained in 10 μl. of shock fluid (1.3 μg protein) was inhibited 20%, 50% and 90% by 0.5, 1 and 3 μl. of extract, respectively, both at 22° and 37°.

** Free 5'-nucleotidase is inhibited 70% in the presence of 4 M urea, but this is reversed on 10-fold dilution of the urea. By contrast, the effect of urea on the enzyme-inhibitor complex seems to be irreversible. With UDPGase, inhibition by 4 M urea is only partially reversed on 10-fold dilution (Fig. 2).

The inhibitor for 5'-nucleotidase was not released by osmotic shock or formation of EDTA-lysozyme spheroplasts, and presumably it is located within the cell membrane. Centrifuged extracts of shocked cells were fractionated with ammonium sulfate and chromatographed on a column of DEAE-cellulose.

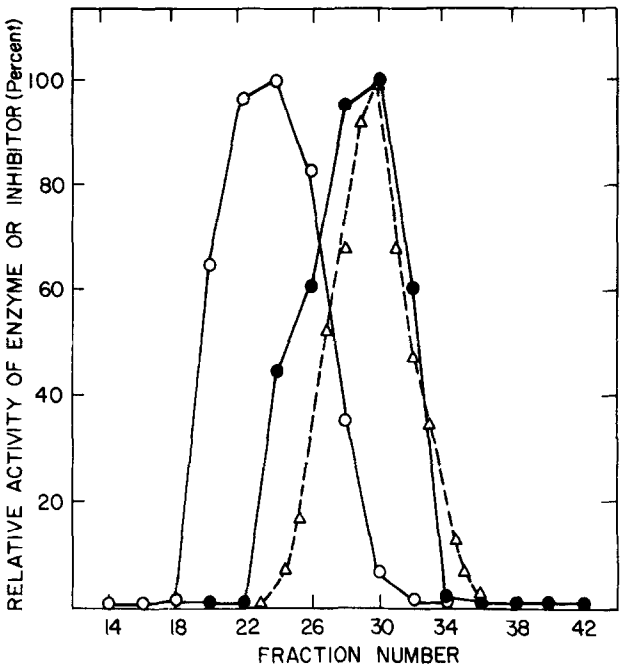

Fig. 3. Separation of 5'-nucleotidase-inhibitor complex and free enzyme on a Sephadex G-100 column. Three ml of a centrifuged sonic extract of E. coli (7 mg protein per ml) were layered on a Sephadex G-100 column (2 x 50 cm), previously equilibrated with 0.03 M Tris-HCl containing 0.01 M $MgCl_2$ and 0.001 M EDTA. Material was eluted with the same buffer. Fraction volume, 3 ml; flow rate, 33 ml per hour. (-o—o-): Enzyme-inhibitor complex, measured by activity released by activation at 37°. "100%" represents an activity of 15.6 units per ml after activation.
(-●—●-) Free inhibitor. "100%" represents 3.1 inhibitor units per ml, measured by the ability to neutralize an equivalent number of 5'-nucleotidase units at 22°.
(-Δ—Δ-) Position of free 5'-nucleotidase, using 3 ml of shock fluid in a separate experiment. "100%" represents an activity of 31 units per ml.

An inhibitor fraction was obtained in 60% yield, but with only a 4-fold purification. However, it was free both of inhibitor-enzyme complex and free enzyme. Fig. 3 indicates that Sephadex G-100 gives a partial separation of complex and inhibitor. The inhibitor was unaffected by treatment for 60 minutes at 22° with 10 μg pancreatic RNase or DNase per 3 mg protein but was inactivated by 200 μg

pronase under similar conditions. It is unlikely that the effect of inhibitor on 5'-nucleotidase is indirect by complexing with essential metals, for a large excess of Ca^{++} and Co^{++} is present in the assay.

So far, we have not been able to separate the inhibitor activities for 5'-nucleotidase and UDPGase. In the absence of bound enzyme, and in 0.03 M Tris, pH 7.3, containing 280 μg protein per ml, the inhibitor activity for 5'-nucleotidase was stable for 10 minutes at 37° but was 90% destroyed at 46°. Destruction of UDPGase inhibitor activity was virtually complete in 10 minutes at 46°. It has not been possible to titrate the inhibitor with each enzyme separately because, after a 200 to 300-fold purification the UDPGase fraction still contains large amounts of 5'-nucleotidase. In fact, superimposable peaks of activity emerged from DEAE and DEAE-Sephadex columns. However, preparations of 5'-nucleotidase are available with only 1% UDPGase activity.

No inhibitor was detected for two other enzymes released by osmotic shock, namely, cyclic phosphodiesterase and the acid phosphatase. Further efforts to separate the inhibitor activities and to determine their function are under way.

REFERENCES

1. Swartz, M. N., Kaplan, N. O., and Lamborg, M. F., J. Biol. Chem., 232, 1051 (1958).
2. Kern, M., and Natale, R., J. Biol. Chem., 231, 41 (1958).
3. Melo, A., and Glaser, L., Biochem. and Biophys. Research Communs., 22, 524 (1966).
4. Swartz, M. N., Kaplan, N. O., and Frech, M. E., Science, 123, 50 (1956).
5. Neu, H. C., and Heppel, L. A., J. Biol. Chem., 240, 3685 (1965).
6. Nossal, N., and Heppel, L. A., J. Biol. Chem., 241, 3055 (1966).