NADPH ACTIVATION OF DEOXYCYTIDYLATE KINASE IN RAT LIVER EXTRACT: INVOLVEMENT OF AN ENDOGENOUS DISULFIDE REDUCTASE SYSTEM

Shuhei Kobayashi and Kazuko Kanayama

Kanazawa Medical University, Department of Biochemistry

Uchinada, Ishikawa-ken, 920-02 Japan

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SUMMARY: Deoxycytidylate kinase (EC 2.7.4.14) in cell-free extract of rat liver, whose activity was highly dependent on the presence of high concentrations of thiol compound during incubation prior to assay, could be activated by substituting 0.1 to 0.2 mM NADPH for the thiol. Partial purification of the extract resulted in a separation of components indicating that the NADPH-dependent activation system was composed of, other than kinase itself, at least two protein factors: one was heat-stable and the other was indistinguishable from NADPH-dependent disulfide reductase [4]. Similarity of this system to thioredoxin-thioredoxin reductase system [7] is noted.

INTRODUCTION: dCMP kinase[1] catalyzes the phosphorylation of dCMP, CMP and UMP, three of the major biosynthetic reactions supplying nucleic acid precursors in mammals [1]. While isolating active enzyme from calf thymus, Sugino *et al* found that the enzyme activity was highly dependent on the presence of thiol compounds, and inactivated enzyme could be reactivated by prior incubation with 2-mercaptoethanol or GSH in a fully reversible manner [1]. In approaching physiological significance of this phenomenon, GSH has been postulated as a candidate for natural activator because of its abundance in animal cells. However, concentration of GSH required for *in vitro* activation of the enzyme was of far above physiological (50 mM), even under continuous generation of GSH with glutathione reductase. While testing various sulfhydryl compounds for the activating effect, we found a highly effective model system for the activation of dCMP kinase, in which a low concentration (10^{-5} M) of *E. coli* thioredoxin coupled with thioredoxin reductase and NADPH successfully activated thymus enzyme [2]. The finding had prompted us to iso-

[1] Abbreviations: dCMP kinase, deoxycytidylate kinase; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione.

late a corresponding system of homogeneous origin, and results supporting the presence of an effective activation system in rat liver extract are presented in this report.

ENZYME ASSAYS: Activity of dCMP kinase was measured radiochemically according to Sugino et al [3] after prior activation as described in the text. To detect dCMP kinase activity in the absence of activator proteins, prior activation was carried out in the presence of 10 mM DTT. During purification of F1 or F3 (see Enzyme preparations), fractions were assayed for their ability to activate dCMP kinase in the presence of NADPH, plus an excess of one fraction and a limiting amount of the other. F2 (see Enzyme preparations) was used as the kinase source. Incubation was for an hour at 37°. Method for measuring disulfide reductase activity was that described by Tietze [4], in which NADPH-dependent reduction of DTNB was followed spectrophotometrically. Measurement of thioredoxin reductase activity was done by a modified method of that for thioredoxin [5] (Method 2) in the presence of a large excess of the latter protein. Liver thioredoxin was determined according to Engström et al [5] (Method 2). Glutathione reductase activity was measured according to Racker [6].

ENZYME PREPARATIONS: Male Wister rats (150-200 g), fed ad libitum, were used as liver donors. Crude extracts were prepared by homogenization with 3 volumes of 0.05 M Tris acetate, pH 7.5, with an Ultra-Turrax homogenizer (Janke & Kunkel, Germany), followed by centrifugation for 20 min at 10,000 x g. Soluble extract was obtained by further centrifugation of the crude extract for 2 hours at 105,000 x g. For further processing of enzymes, pH of the crude extract was adjusted to 5.0 with acetic acid. The precipitate formed was removed by centrifugation for 20 min at 10,000 x g. The supernatant solution was adjusted to pH 7.5 by addition of 1 N NH_4OH to give pH 5 supernatant, which contained all of protein factors for the activation of dCMP kinase including the kinase itself. After ammonium sulfate precipitation, however, two fractions were required for the activity. The fraction precipitating at 50-65 % saturation (AS50-65) after passing through a column of Sephadex G-25, was applied onto a column of DEAE cellulose. The absorbed protein was eluted out with a linear gradient approaching 0.5 M KCl (see legend to Fig. 2), and fractions containing dCMP kinase-activating principle were pooled (F1). The fraction precipitating at 65-90 % ammonium sulfate saturation (AS65-90) was purified by heat treatment at 53° for 3 min, followed by gel filtration with a Sephadex G-25 column and gradient elution from DEAE cellulose. The last treatment resulted in a separation of fractions containing dCMP kinase (F2) from the second activator protein (F3). E. coli thioredoxin was isolated and purified according to the method described by Laurent et al [7]. E. coli thioredoxin reductase was purified according to Moore et al [8].

RESULTS: When either pH 5 supernatant, after dialysis, or the soluble extract was incubated in the presence of NADPH prior to dCMP kinase assay, a remarkable activation was observed (Table I), while virtually no activity was detected either in the enzyme solution which had been incubated without NADPH or in that assayed without prior incubation. The extent of activation

TABLE I

Effects of Nicontinamide Nucleotides and Thiol Compounds on Activation of dCMP Kinase in Liver Extracts.

Expt. No.	Condition for Prior Incubation	dCMP Kinase Activity
		nmol/h/tube
1	No incubation	0.6
	+ 3.3 mM NADPH	36.9
2	No incubation	0.5
	No addition	0.5
	+ 0.17 mM NADPH	13.3
	+ 0.83 mM NADPH	15.7
	+ 0.17 mM NADP	2.7
	+ 0.17 mM NADH	1.5
	+ 0.17 mM NAD	0.9
	+ 9.2 mM DTT	12.5
	+ 3.3 mM GSH	1.2
	+ 33.3 mM GSH	6.7

Either pH 5 supernatant, after dialyzing overnight against 0.05 M Tris acetate, pH 7.5 (Expt. 2), or soluble extract (Expt. 1) was incubated at 37° for 1 hour. The incubation was carried out under conditions listed above, then 50 µl portions were taken from the mixtures and dCMP kinase activity was measured.

with NADPH even exceeded the activity that was attained by incubating with DTT, the most effective thiol of low molecular weight so far tested. In contrast, only 60 % activation was observed with GSH at a concentration as high as 33 mM. A much lower activity was detected at its concentration (3.3 mM) which was considered to be physiological. Incubation with NADH or NADP in place of NADPH was far less effective. NAD had no activity at the same range of concentration. Fig. 1 shows the time dependency of the dCMP kinase activation by NADPH. The result also indicated that the activating effect was exerted during the prior incubation and was not due to direct stimulation of

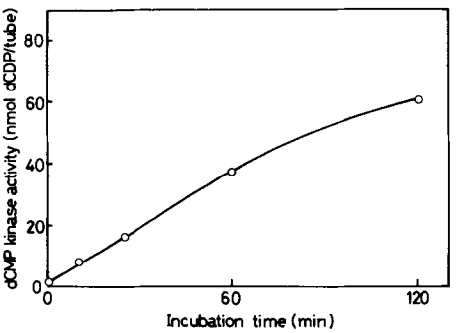

Figure 1. Activation of dCMP kinase in rat liver extract by NADPH.

The soluble extract was incubated at 37° in the presence of 3.3 mM NADPH in a total volume of 1.0 ml. At each interval, 50 µl portion was taken and the enzyme activity was measured as described under "Enzyme assays".

the kinase reaction. The product of the kinase reaction thus obtained was identified as dCDP by high voltage paper electrophoresis at pH 3.5 as well as by paper chromatography in isobutyric acid-1 N NH_4OH-0.4 M EDTA (100:60:0.4). Fractionation of the pH 5 supernatant with ammonium sulfate resulted in a separation of two protein fractions, AS50-65 and AS65-90, both of which were required for dCMP kinase activity on incubation with NADPH prior to the kinase assay. AS50-65 contained both NADPH-dependent disulfide reductase and glutathione reductase, while AS65-90 was found to contain the kinase protein. On purification of each of the ammonium sulfate fractions for activator proteins as described under "Enzyme preparations", two protein fractions, F1 and F3, were obtained from AS50-65 and AS65-90, respectively. Table II illustrates the requirement for both of the purified fractions to activate dCMP kinase in the presence of NADPH. Neither fraction alone (F1 or F3) gave any dCMP kinase activity, while both together gave an activity over half that seen with E. coli thioredoxin reductase system. After fractionation of AS50-65 through DEAE cellulose column, each of the resulting fraction was analyzed for protein, glutathione reductase and NADPH-dependent disulfide reductase activities, as well as for the capacity to activate dCMP kinase in the presence of NADPH and F3 (Fig. 2). At least three major peaks exhibiting the disulfide

TABLE II

Effects of Purified Protein Fractions on Activation of dCMP Kinase.

Addition	dCMP Kinase Activity
	nmol/h/tube
None	0.0
F1 (80 μg)	0.3
F3 (10 μg)	0.2
F1 (80 μg) + F3 (10 μg)	7.0
F1 (80 μg) + F3 (10 μg) - NADPH	0.3
F1 (80 μg) + F3 (10 μg) - dCMP kinase	0.1
E. coli thioredoxin (35 μg) + *E. coli* thioredoxin reductase (18 μg)	12.4
No prior incubation	0.1

To each tube containing 0.17 mM NADPH, 20 μg of dCMP kinase (F2), 5 mM Tris acetate, pH 7.5 and 1mM EDTA were added components listed above. Total volume was 0.2 ml. The mixtures were then incubated at 37° for 1 hour. Thereafter, 50 μl portions were taken from the mixtures and dCMP kinase activity was measured under "Enzyme assays".

reductase activity were seen, and a close coincidence was observed between the third peak (P3) of disulfide reductase activity and the dCMP kinase activating principle, whereas glutathione reductase was well separated from the latter protein. Besides, each of fractions from the DEAE cellulose column was also tested for thioredoxin reductase activity (data not shown), which coincided only with the third peak (P3) of disulfide reductase activity.

DISCUSSION: The present study indicated that at least two protein components were required other than NADPH and dCMP kinase itself for activation of the latter. One of these components, which was localized in F3, might correspond to liver thioredoxin [9], since 1) both were eluted out from DEAE cellulose with an identical ionic strength (0.15 M KCl), 2) both of their activities were resistant to heat treatment (90°, 5 min), and 3) they had similar

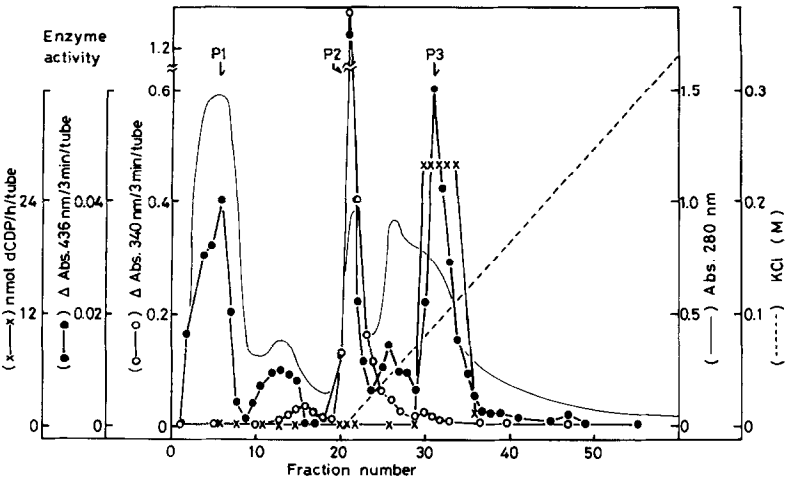

Figure 2. Chromatography of AS50-65 on DEAE cellulose.

AS50-65 (2.0 ml) was passed through a column of Sephadex G-25 (2.6 x 40 cm). The fractions containing dCMP kinase-activating principle were then pooled (22 mg protein, in 19 ml) and applied to a column of DEAE cellulose (1.3 x 8 cm) previously equiliburated with 5 mM potassium phosphate, pH 7.0 containing 10^{-4} M EDTA (Starting buffer). After washing the column with 50 ml of Starting buffer, a linear gradient of KCl (0 to 0.5 M, in 100 ml each of Starting buffer) was established and 2.5 ml fractions were collected. Samples were taken from each fraction and assayed for NADPH-dependent disulfide reductase (●-●) and glutathione reductase (o-o) activities. For measurement of dCMP kinase-activating principle (x-x), 50 µl portion of each fraction was incubated at 37° for 1 hour in the presence of F3 (50 µl, 15 µg), dCMP kinase (F2) (50 µl, 25 µg) and NADPH (3.3×10^{-4} M), thereafter 50 µl portion was taken from the mixture and dCMP kinase activity was measured. All of these assay methods were as described under "Enzyme assays" except that for the activating principle, in which not an excess amount of F3 was used because of limited supply of the latter protein.

molecular weight to each other of about 12,000 daltons judged by a Sephadex G-75 gel filtration (data not shown). The other component was indistinguishable from one of the NADPH-dependent disulfide reductases in the chromatographic pattern (Fig. 2) as well as from thioredoxin reductase. Although scantiness of description on mammalian thioredoxin reductase made it difficult to compare properties of these reductase proteins in detail, a highly purified enzyme preparation was presently available in our laboratory, and it retains full capacity for NADPH-dependent reduction of both DTNB and liver thioredoxin as well as that for activating dCMP kinase in the presence of

liver thioredoxin and NADPH (report in preparation). The identity has not yet been determined with their capacity to act as hydrogen donor for the ribonucleotide reduction; however, results obtained from model experiments using E. coli [2] or tumor [10] system strongly suggested the occurence of an endogenous thioredoxin-thioredoxin reductase system-mediated activation of the liver kinase, probably via reduction of essential SH group(s) on the kinase molecule. Moreover, the presence of thioredoxin in rat liver has been confirmed [9].

At present no direct evidence is available in favor of its active role in regulating SH-dependent enzymes such as dCMP kinase in vivo. However, the demonstration of the ability of endogenous disulfide reductase system to activate dCMP kinase in the presence of a "physiological" concentration of NADPH would be favorable for the postulation. GSH had long been thought to be the major thiol which maintains sulfhydryl-containing enzymes in the active state. Our finding also suggests that this disulfide reductase system plays a similar role more efficiently and possibly, in a more specific manner.

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