METAL ION COMPLEXES OF CYTIDINEDIPHOSPHOCHOLINE

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Received December 19,1975

SUMMARY

Cytidine-5'-diphosphocholine (CDPcholine) forms a complex with Mg^{2+} and Mn^{2+} ions as indicated by the broadened ^{31}P NMR peaks of CDPcholine in the presence of these ions. Additional evidence for the complex is the decrease in absorption at 360 nm when CDPcholine is added to solutions of 8-hydroxyquinoline and Mg^{2+}. The stability constant of the Mg-CDPcholine complex was found to be 20 M^{-1}.

All known phosphotransferases, with one exception, require a divalent metal ion for activity (1). Metal ions form complexes with adenosine triphosphate and the complex is the actual substrate for reactions catalyzed by certain kinases. The formation of Mg-ATP has been demonstrated with NMR spectroscopy (2,3) and the stability constant of the complex determined (4,5). This report provides evidence for the formation of a metal-CDPcholine complex. This complex might be the substrate for cytidine-5'-diphosphocholine: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2).

EXPERIMENTAL PROCEDURE

^{31}P NMR spectra (3,6) of 0.5 M CDPcholine solutions were obtained in the presence and absence of divalent metal ions. $MgSO_4$ and $CaSO_4$ concentrations were 0.5 M and the concentration of $MnSO_4$ ranged from 5×10^{-6} to 1×10^{-4} M. The pH of the solutions was 8.5 and the temperature was 22 ± 1°C. Spectra were obtained with a Varian Associates HA-60IL NMR spectrometer at 24.3 MHz. Sample tubes of 3 mm diameter were used with sample sizes varying from 0.3 ml to 0.5 ml. All chemical shifts were measured relative to 85% phosphoric acid, which was used as an external standard. Spectra were calibrated using standard side band techniques.

The stability constant of the Mg-CDPcholine complex was determined by a method used to measure the stability constants of the Mg^{2+} complexes

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of ATP and ADP (4,5,7). The method is based on the competition for magnesium ion between the ligand being investigated and 8-hydroxyquinoline, since the concentration of the Mg-8-hydroxyquinoline complex is conveniently determined spectrophotometrically at 360 nm (a_{360} = 2300 M^{-1} cm^{-1}) (4). In the present experiments, the concentration of 8-hydroxyquinoline was 1 mM, CDPcholine was 5, 10, 20 and 40 mM in four independent experiments, and $MgCl_2$, which was added by microliter syringe or pipet from a 0.05 M stock solution, varied from zero to 1.2 mM in each experiment. The solution also contained 0.05 M tris-(hydroxymethyl)-aminomethane (Tris) buffer, pH 8.5. All absorption measurements were made at 360 nm with an Hitachi Perkin-Elmer Spectrophotometer Model 139. The temperature of the liquid in the sample cells was maintained at 37.0 ± 0.2°C by circulating water through the bottom of the cell holder compartment.

NMR data from the study of manganese ion effects on chemical shift and peak width were subjected to analysis of variance and Duncan's multiple range test. Data from the study of Ca^{2+} and Mg^{2+} effects were analyzed by Student's t-test.

RESULTS AND DISCUSSION

The ^{31}P NMR spectrum of CDPcholine had a single absorption peak whether metal ions were present or not. The chemical shift (δ) was 11.8 ppm upfield from the H_3PO_4 reference (Tables 1 and 2). This agrees, as expected, with the value reported for the α-phosphorus of ATP and ADP (δ = 11 ppm) (4). Changing the pH from 7 to 12 did not change the value of δ, nor did the presence of Mn^{2+} or Ca^{2+} (Tables 1 and 2). These data also agree with data reported for the α-phosphorus of ATP but not ADP (3). When $MgSO_4$ was added there was a small, but significant ($P<.005$) change in the chemical shift (Table 2).

Since metal ions broaden NMR peaks of the nuclei of compounds with which they form complexes by increasing relaxation times, peak widths were also measured. The observed sequential increase in peak widths with increasing Mn^{2+} concentration (Table 1) indicates the formation of a complex between this metal ion and CDPcholine. Peak widths at all concentrations of $MnSO_4$ were significantly different from the control, which lacked $MnSO_4$ ($P<.05$ or $P<.01$ as shown in Table 1). Peak widths at each concentration greater than 1 x 10^{-5} M were also significantly different from peak widths at all other concentrations ($P<.01$). This is consistent with data reported for ATP (3). A significant change in the peak width was

TABLE 1

^{31}P - NMR of CDPcholine with Manganese Ions[a]

Manganese Ion Concentration	Chemical Shift (ppm)	Width of Peak (ppm)[b]
0	11.8 ± .1 (9)[c]	.85 ± .10 (8)[c]
5×10^{-6} M	12.0 ± .1 (4)	1.08 ± .08 (4)[d]
1×10^{-5} M	12.0 ± .1 (4)	1.23 ± .07 (5)[e]
2×10^{-5} M	12.0 ± .2 (3)	1.51 ± .10 (5)[e]
5×10^{-5} M	11.7 ± .1 (4)	2.07 ± .10 (5)[e]
1×10^{-4} M	11.9 ± .2 (5)	3.02 ± .20 (4)[e]

[a]The CDPcholine concentration was .5 M, pH 8.5, and $MnSO_4$ was present at the concentration shown. Other conditions are described in the text.

[b]Measured at ½ height.

[c]Mean ± standard deviation. The number of observations is given in parentheses.

[d]Significantly different from control ($P<.05$).

[e]Significantly different from control ($P<.01$).

TABLE 2

^{31}P - NMR of CDPcholine with Magnesium and Calcium Ions[a]

Metal Ion	Chemical Shift (ppm)	Width of Peak (ppm)[b]
None	11.8 ± .1 (9)[c]	.85 ± .10 (8)[c]
Mg^{2+}	12.2 ± .1 (4)[d]	1.02 ± .07 (4)[e]
Ca^{2+}	11.9 ± .1 (4)	.77 ± .03 (4)

[a]The CDPcholine concentration was .5 M, pH 8.5, and, where indicated, $MgSO_4$ or $CaSO_4$ was present at .5 M. Other conditions are described in the text.

[b]Measured at ½ height.

[c]Mean ± standard deviation. The number of observations is given in parentheses.

[d]Significantly different from control ($P<.005$).

[e]Significantly different from control ($P<.05$).

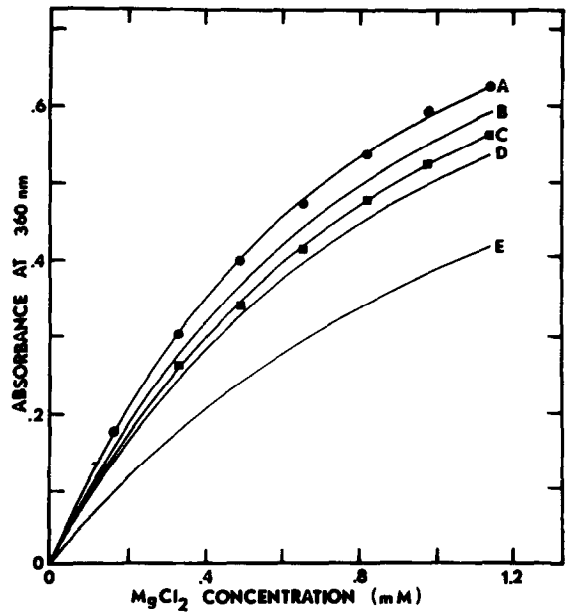

FIGURE 1. Absorbancy of the Mg^{2+} complex of 8-hydroxyquinoline in the presence and absence of CDPcholine. Solutions contained 50 mM Tris buffer, pH 8.5, 1 mM 8-hydroxyquinoline, $MgCl_2$ as shown, and either no CDPcholine (●) or 20 mM CDPcholine (■). The other conditions are described in the text. See text for a description of the curves.

also caused by the diamagnetic Mg^{2+} ion at equimolar concentration ($P<.05$, Table 2), but there was no change when $CaSO_4$ was added ($P<.05$). High resolution NMR would be needed to distinguish between partial separation of the two phosphorus peaks and increased relaxation time as a cause of the peak broadening. The former would indicate unequal interaction between each of the two phosphates and the metal ion.

To determine the stability constant of the Mg-CDPcholine complex, the absorption of a solution containing 1 mM 8-hydroxyquinoline was measured at several different concentrations of $MgCl_2$ from zero to 1.2 mM. These results are shown in Figure 1 (circles, Curve A). The inclusion of CDPcholine at a concentration of 20 mM in the 8-hydroxyquinoline solution resulted in a lower absorbance for each concentration of $MgCl_2$ (Figure 1, squares). This is interpreted as the result of the formation of a Mg-CDPcholine complex,

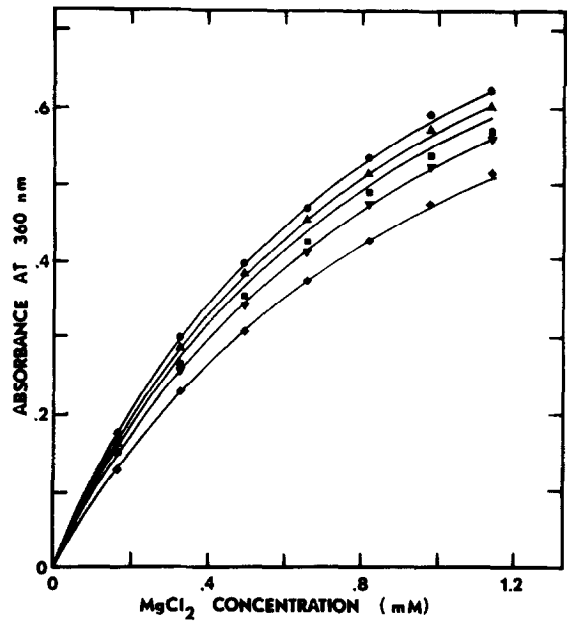

FIGURE 2. Absorbancy of the Mg^{2+} complex of 8-hydroxyquinoline at various concentrations of Mg^{2+} and CDPcholine. The solutions were the same as described for figure 1 except ● = no CDPcholine; ▲ = 5 mM CDPcholine; ■ = 10 mM CDPcholine; ▼ = 20 mM CDPcholine; and ◆ = 40 mM CDPcholine. The curves were calculated assuming a value of K_s = 20 M^{-1} (see text).

with correspondingly less Mg^{2+} available for binding by 8-hydroxyquinoline. The decrease in absorbance is related to the stability of the Mg-CDPcholine complex. The curves B, C, D, and E in Figure 1 are theoretical absorption curves calculated from assumed stability constants of 10, 20, 30, and 100 M^{-1}, respectively. The agreement between the data points (■) and curve C in Figure 1 indicates that the stability constant is near 20 M^{-1}. Figure 2 shows data obtained at various CDPcholine concentrations. The curves were calculated for each of the CDPcholine concentrations assuming a value of 20 M^{-1} for the stability constant. The general agreement of the experimental data with the theoretical curves gives additional support for this value for the stability constant of the Mg-CDPcholine complex.

Cholinephosphotransferase requires either Mg^{2+} or Mn^{2+} for activity (8). The addition of Ca^{2+} even in the presence of Mg^{2+} or Mn^{2+} is inhibitory

(8). Since CDPcholine forms complexes with both Mg^{2+} and Mn^{2+} ions but not with Ca^{2+}, it may be the metal-CDPcholine complex which is the substrate for the enzyme. Although the stability constant of the Mg-CDPcholine complex is quite low, the intracellular concentration of magnesium is high; therefore, the intracellular concentration of Mg-CDPcholine could be higher than that of free CDPcholine. The low stability constant for the complex would allow regulation of phospholipid biosynthesis by changes in concentration of magnesium within the endoplasmic reticulum.

ACKNOWLEDGMENT

This research was supported by Public Health Service Grant HE-02615.

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