Formycin 5’-triphosphate, a fluorescent analog of ATP, as a substrate for adenylate cyclase

(3’,5’-cyclic formycin monophosphate synthesis/high-pressure liquid chromatography/radioimmunoassay calibration with cyclic formycin monophosphate/stimulation by guanyl-5’-yl imidodiphosphate/rat osteosarcoma)

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ABSTRACT Formycin 5’-triphosphate (FoTP), a fluorescent analog of ATP, is shown to be a substrate for the membrane-bound adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] from rat osteosarcoma cells. The formation of the adenylate cyclase reaction product, 3’,5’-cyclic formycin monophosphate (cFoMP), was followed by the conventional radioimmunoassay (RIA) procedure used to detect cAMP and by an assay procedure in which the reaction product was separated from the substrate by reverse-phase high-pressure liquid chromatography (HPLC) and the reaction product was detected by fluorometry. Because the HPLC-fluorometric procedure can determine the amount of cFoMP present in the reaction mixture within 6 min, the enzymatic conversion of FoTP to cFoMP can be followed directly during the course of a typical 15-min incubation. The amount of cFoMP detected by this procedure was found to be within 2% of the values obtained by the RIA. The rate of product formation with FoTP was similar to that observed with ATP and the activity of the enzyme was enhanced about 5-fold with guanyl-5’-yl imidodiphosphate when either ATP or FoTP was used as the substrate. Kinetic studies revealed values for the $V_{max}$ of 120 pmol/min per mg of protein and apparent $K_m$ values of 220 $\mu$M with both substrates. In addition to suggesting that the recognition of the substrate by the adenylate cyclase may not require a specific chemical structure of the 5-membered ring of the base or a unique configuration about either the glycosyl or the C(5')-C(4') bond, the results of this study are consistent with the idea that the cytotoxicity observed with the adenine analog formycin may be the result of its metabolism to cFoMP. Furthermore, these studies indicate that the fluorescent analog FoTP can be used, in combination with HPLC, to provide an alternative, nonradioactive direct method for the assay of adenylate cyclase catalytic activity.

Formycin (Fig. 1), an analog of adenosine, has been shown to be cytotoxic in a number of mammalian systems (1, 2), to have some antiviral activity (3), and to inhibit several aspects of purine metabolism (4). Because an understanding of the biochemical basis for this toxicity requires information on the metabolism of formycin after its incorporation, a number of studies have examined the fate of this analog. For example, in vitro studies have shown that formycin is a substrate for adenosine kinase (5, 6) and adenosine deaminase (5, 7). In addition, an in vitro study showed the conversion of formycin to formycin 5’-triphosphate (FoTP) (8), and in vitro studies showed that nucleic acids containing formycin are formed from FoTP (9). However, an alternative fate is available for FoTP; namely the formation of 3’,5’-cyclic formycin monophosphate (cFoMP). Although one report suggests that cFoMP may be formed in vitro by mouse leukemia cells (10), direct evidence that FoTP is a substrate for adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in vitro has not been reported.

In addition to being the subject of investigations on their metabolic fate, formycin nucleotides should be potentially useful probes of the mechanism of a number of reactions because, as mentioned above, they are accepted as substrates by a variety of enzymes that show high specificity and because, as shown in Fig. 1, the structural differences between formycin and adenosine appear to be restricted to the atoms located at the C-8 and N-9 positions. However, x-ray diffraction studies on crystals of the nucleosides indicate differences in the three-dimensional structure as well. These differences include not only the conformation about the glycosyl bond but also the conformation about the C(5’)—C(4’) bond (11). Although only tentative conclusions can be drawn about the structures of FoTP in solution from studies of formycin crystals, there is some evidence that the anomalous structure of formycin exists in its derivatives in solution. For example, Ward and Reich (12) proposed that most of the unusual properties of polymers of formycin 5’-monophosphate (FoMP) could be explained by considering that the conformation about the C-glycosyl bond is syn and not anti as in the case with adenosine compounds (12) and Schramm and coworkers (13) have explored the mechanism of action of AMP nucleosidase with FoMP.

Finally, mechanistic studies are facilitated by the fact that this analog is fluorescent, with excitation and emission maxima of 295 nm and 340 nm, respectively (14). Because this fluorescence is often enhanced upon binding to proteins, FoTP has already been used for the study of the interaction of proteins with nucleotides by photodissociation (15).

**Abbreviations:** FoMP, FoDP, and FoTP, formycin 5’-mono-, di-, and triphosphate, respectively; cFoMP, 3’,5’-cyclic formycin monophosphate; cAMP, 3’,5’-cyclic adenosine monophosphate; HPLC, high-pressure liquid chromatography; p[NH]ppG guanyl-5’-yl imidodiphosphate; RIA, radioimmunoassay.
been used to study the mechanism of the ATPase of myosin (15) as well as a Na+,K+-ATPase (16) by using stopped-flow fluorometry.

We report here an investigation to determine whether FoTP is a substrate for the adenylate cyclase activity of rat osteosarcoma membranes in order to gain a further understanding of the cytotoxic effects of formycin and to assess the potential of the fluorescence of formycin nucleotides in studying the mechanism of cyclic nucleotide formation by adenylate cyclase and for future studies on the binding of cyclic nucleotides to proteins. The reaction of FoTP is followed by utilizing its fluorescence in conjunction with high-pressure liquid chromatography (HPLC) procedure previously developed for adenosine nucleotides (17).

**EXPERIMENTAL PROCEDURES**

**Materials.** Formycin A was obtained from Meiji Seika Kaisha Ltd., Kawasaki, Japan. ATP, AMP, 3’,5’-cyclic AMP (cAMP), dithiothreitol, creatine phosphate, creatine kinase, and 3’,5’-cyclic nucleotide phosphodiesterase (beef heart) were from Sigma. Methanol (spectral grade) was from Waters. The radioimmunoassay (RIA) for cyclic nucleotide determinations was from New England Nuclear. Guanyl-5’-yl imidodiphosphate (p[NH]ppG) was from ICN.

**Synthesis of Formycin Nucleotides.** FoMP and FoTP were prepared by using methods similar to those previously used (18) for synthesis of 6-thioguanosine nucleotides, and cFoMP was prepared from FoMP essentially by the method of Smith et al. (19) for the preparation of cAMP. Concentrations of formycin nucleotides were determined spectrophotometrically using ε<sub>295</sub> = 10,600 liter mol<sup>-1</sup> cm<sup>-1</sup> at pH 7.0 (14).

**Preparation of Plasma Membranes.** The rat osteosarcoma (obtained from the Papanicolaou Cancer Research Institute) was maintained by subcutaneous transplantation in the rat dorsal hind quarter. Tissues, obtained from 18- to 30-day tumors, were homogenized and a crude membrane fraction (P-47) was obtained after centrifugation of the homogenate at 47,000 x g. Membranes were further purified by density gradient centrifugation. Additional methods have been described (20).

**Assay for Adenylate Cyclase Activity.** Assays were performed in the following incubation medium: 25 mM Tris-HCl at pH 7.5, 2 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 0.3 mM nucleoside triphosphate, and a nucleoside triphosphate-regenerating system consisting of 7 mM creatine phosphate and 5 units of creatine kinase in a total reaction volume of 0.1 ml. Separate experiments showed that FoTP and formycin 5’-diphosphate (FoDP) were substrates in this regenerating system. The reaction was initiated by addition of up to 200 µg of membrane protein in 10 µl of 25 mM Tris-HCl, pH 7, to a final volume of 100 µl. Reaction mixtures were incubated at 30°C, the reactions were terminated by addition of 1 ml of 6% trichloroacetic acid, and the precipitate was removed by centrifugation at 30,000 x g for 15 min. Supernatant fractions were purified further by passage through a Dowex (AG 50W-X4) column (0.8 x 2 cm) and the cyclic nucleotides were eluted with water. Assay blanks containing no membrane protein were processed similarly. Fractions containing cyclic nucleotides were lyophilized and the amount of cAMP or cFoMP present in their respective column-purified fraction was determined.

HPLC assays of cyclic nucleotide levels were performed as described (17), on a Waters ALC/GPC 204 liquid chromatograph. The column used was a 4 mm x 30 cm µBondapak C<sub>18</sub>, 10-µm particle size, from Waters, protected by a guard column containing pellicular Corsil. The mobile phase consisted of 10 mM potassium phosphate, pH 5.5, with 10% (vol/vol) methanol. A flow rate of 2 ml/min was maintained and all separations were at room temperature. Formycin nucleotides were detected by fluorescence with a Schoeffel FS970 grating spectrophotometer. Excitation was obtained with light at 300 nm, using a 7-54 bandpass filter. Emission was obtained with a 320-nm cut-off filter. Adenine nucleotides were detected at 254 nm with a Waters model 440 absorbance detector.

RIAs, patterned after previously described methods (21), were performed in disposable glass culture tubes (12 x 75 mm). Reagent solutions used in the RIA were reconstituted, following instructions provided by supplier, with 50 mM sodium acetate, pH 6.2, and the pH of all reconstituted solutions was checked and, if necessary, adjusted to 6.2 with 0.1 M acetic acid. Samples to be assayed (100 µl in 50 mM sodium acetate, pH 6.2) each received 100 µl of solution containing <sup>125</sup>I-labeled 2’-O-succinyl-cAMP tyrosine methyl ester (approximately 10,000 cpm) together with normal rabbit serum (both solutions diluted or reconstituted as directed by supplier) followed by 100 µl of cAMP antiserum (reconstituted as directed). The tubes were covered, stirred with a Vortex mixer, and placed in a cold room (4°C) for 16-18 hr. On the next day each tube received 1 ml of cold 50 mM sodium acetate, pH 6.2 and the antibody precipitates were harvested by centrifugation (4000 x g for 15 min) in a refrigerated Sorvall model RC2-B centrifuge equipped with a model GSA rotor and a model 00388 12 place carrier. The supernatants were removed by aspiration. The radioactivities in the precipitates were measured in a gamma spectrometer. Individual standard curves were generated for both cAMP and cFoMP. Each sample was assayed in duplicate. In the absence of any added cyclic nucleotide about 45-50% of the radioactivity was precipitated.

**RESULTS**

**Calibration of the RIA with cFoMP.** The RIA method for the determination of cAMP utilizes the cAMP concentration dependence of the displacement of <sup>125</sup>I-labeled 2’-O-succinyl-cAMP tyrosine methyl ester from a specific antibody. We investigated the concentration dependence of cFoMP in this system to determine whether or not cFoMP binds to the antibody and to construct a calibration curve for measuring cFoMP concentration. Fig. 2 shows that cFoMP does displace the radioactivity.
active analog of cAMP from the antibody, although higher concentrations of cFoMP are required for the same amount of displacement compared to cAMP. These data are consistent with cAMP binding to the antibody approximately 4.5-fold more strongly than cFoMP does. Therefore, cFoMP can be measured by this method, although a calibration curve using authentic cFoMP is required. It should also be noted that with cFoMP the limit of detection appears to be about 2 pmol.

Adenylate Cyclase Activity with FoTP. The membrane-bound adenylate cyclase activity from the rat osteosarcoma has been characterized by using [α-32P]ATP as substrate and monitoring the formation of [32P]cAMP (20). This activity has been shown to be stimulated about 5-fold with 5 μM p[NHppG (20). On the basis of those previous results, and assuming that FoTP was as good a substrate as ATP, we could expect about 15 pmol of cyclic nucleotide would be formed, after 10 min with 10 μg of membrane protein, an amount easily detected by the RIA (see Fig. 2). Starting with a total reaction volume of 100 μl, the injection of only 50 μl onto the HPLC column should contain enough cyclic nucleotide, about 7.5 pmol, to be detected in the fluorometric assay, as can be seen in the calibration curve shown in Fig. 3.

In Fig. 4 we show the results of a representative experiment in which osteosarcoma membranes (10 μg of membrane protein) were preincubated with 5 μM p[NHppG and the mixture was added to a solution containing 0.3 mM FoTP. At suitable times, samples were removed and processed for analysis of cFoMP content by using both fluorometry and the RIA. The analysis of the samples by HPLC-fluorometry showed a peak with a retention time of 3.5 min identical to that expected for cFoMP. The area under this peak increased with reaction time: no peak was detectable in a sample taken 30 seconds after the start of the reaction (Inset A of Fig. 4), and a peak with this retention time was clearly seen after 15 min of incubation (Inset B of Fig. 4). Similar profiles were obtained after the injection of samples of the incubation mixture directly onto the HPLC column, indicating that prior processing of the samples is not required for analysis. No peak was observed if the osteosarcoma membranes were omitted or if they were boiled prior to the start of the reaction, thus ruling out the possibility of the formation of cFoMP by a nonenzymatic process. In one series of experiments, the component with a retention time of 3.5 min was collected as it emerged from the HPLC column. After incubation with commercially available 3',5'-cyclic phosphodiesterase, a sample was reinkjected onto the HPLC column. The fluorometric tracing showed one major peak (over 80%) with a retention time of 2 min, identical to FoMP, and a minor peak (about 20%) with a retention time of 3.5 min. These results indicate the reaction product was degraded by 3',5'-cyclic phosphodiesterase to FoMP, a result consistent with the conclusion that it was cFoMP.

Finally, these same samples were analyzed for cFoMP content by using the RIA procedure. The values obtained were within 2% of those obtained by fluorometry (Fig. 4).

Comparison of Adenylate Cyclase Activity with FoTP and ATP. The activities of osteosarcoma membranes with FoTP and ATP as substrates were compared. Because the amount of cAMP produced in a reaction is below the level of detection by absorption spectroscopy (see above), the formation of cyclic nucleotide was followed by the RIA only. In experiments without p[NHppG, both FoTP and ATP showed the same rate of cyclic nucleotide formation (Table 1). Increasing the concentration of p[NHppG produced an activation of the cyclase of

![Fig. 3. Calibration of fluorometric detector with cFoMP. Solutions were prepared ranging from 0.4 to 7 μM, and 5 μl of each was injected onto the column. Column, mobile phase, fluorescence, and absorbance conditions were as described in Experimental Procedures. Areas of the cFoMP peaks were determined from tracings obtained at each concentration and are plotted (in arbitrary units) as a function of amount of cFoMP injected. (Inset A) Tracing obtained after injection of 5 pmol of cFoMP. (Inset B) Tracing obtained with 15 pmol of cAMP (arrow indicates retention time of authentic cAMP).](image)

![Fig. 4. Kinetics of cFoMP formation as determined by RIA and fluorometry. Adenylate cyclase activity was determined at 0.3 mM FoTP with 100 μg of membrane protein in a final reaction volume of 100 μl. Reactions were terminated, and cFoMP was purified and then analyzed by RIA or HPLC. (Insets) Representative HPLC profiles obtained at 30 seconds (A) and 15 min (B) after start of the reaction. The arrows indicate the retention time observed after injection of authentic cFoMP. The area under the curves was determined by integration and amount of cFoMP present was determined from a calibration curve of the type shown in Fig. 3. Points represent data obtained from RIA. Data obtained from HPLC assay gave values within the error bars.](image)

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<th>Table 1. Effect of p[NHppG on cyclic nucleotide formation by membrane-bound adenylate cyclase from osteosarcoma</th>
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<td>Cyclic nucleotide formed, pmol/15 min</td>
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<tr>
<td>None</td>
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<tr>
<td>p[NHppG</td>
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<td>0.5 μM</td>
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Membranes (10 μg) were preincubated for 5 min at 30°C with p[NHppG at the concentrations indicated, followed by the addition of the adenylate cyclase reaction mixture. The mixtures were incubated for an additional 15 min at 30°C and the reactions were terminated. Results are mean ± SEM for triplicate experiments.
similar magnitude, with either FoTP or ATP as substrate (Table 1). Increasing the concentration of membrane protein 5-fold produced a corresponding increase in cyclic nucleotide production. Rate determinations of several concentrations of both FoTP and ATP revealed an apparent $K_m$ of the enzyme in the presence of 5 $\mu$M [3H]ppG of 220 $\mu$M and a $V_{max}$ of about 120 pmol/min per mg of protein.

**DISCUSSION**

The adenosine analog formycin is cytotoxic in a number of systems; although it has been shown to inhibit several aspects of purine metabolism (4), its mechanism of action remains unknown. On the basis of results of a previous study (10) it was suggested that the cytotoxicity of formycin may be a result of the formation of cFoMP from FoTP (22). The results of the present study support the speculation (10) that cFoMP can be one of the metabolic fates of FoTP by showing that the membrane-bound adenylate cyclase activity of rat osteosarcoma cells is able to catalyze the conversion of FoTP to cFoMP. In addition, the slower rate of hydrolysis of cFoMP compared to CAMP by beef heart phosphodiesterase that has been observed previously (23) supports our speculation that abnormal levels of this cyclic nucleotide could be accumulated and might be expected to interfere with a number of intracellular regulatory processes such as phosphorylation by aCAMP-dependent protein kinase. While the effect of cFoMP on protein kinase has to date not been studied, the effect of several other aCMP analogs has. Thus, the 3',5'-cyclic monophosphates of tubercidin (24) and 2-fluoro-adenosine (25) have been shown to be as good as aCAMP as activators of protein kinase activity. This finding, together with the observation that the analogs are converted to their corresponding nucleoside 5'-triphosphates and 3',5'-cyclic monophosphates in a variety of lymphoid cell lines, supports the conclusion that formation of cyclic nucleotides may be involved in toxicity.

It should be noted also that the in vivo formation of cyclic nucleotides from 2-fluoroadenosine 5'-triphosphate (26) and tubercidin 5'-triphosphate (27) can provide some insights into defining the essential stereochemical requirements in substrate recognition by adenylate cyclase. Other information derives from studies with analogs and isolated (cell-free) preparations. Analogs with modifications in the triphosphate chain, the ribose, or the purine moiety have been used. The result of findings with the phosphate-modified analogs, including those in which CH$_2$ or NH replaces the $\beta$-$\gamma$ bridging oxygen (28) and those in which sulfur replaces a nonbridge oxygen, have been reviewed (29). Studies with ribose-modified analogs showed that adenine arabinonucleoside 5'-triphosphate (30) was a substrate, suggesting that structural changes at the 2' position of the ribose do not preclude catalysis.

Studies using analogs with modifications in the purine moiety showed that 2-amino purine ribonucleoside 5'-triphosphate was neither a substrate nor an effective inhibitor of the adenylate cyclase from *Brevibacterium liquefaciens*, while some activity was detected with 2-aminoadenosine 5'-triphosphate (31), suggesting that the 6-amino group is required for substrate recognition and that modification at the 2' position may not be crucial. The present results, showing that FoTP is able to replace ATP as a substrate, suggest that the difference in structure in the 5-membered ring between formycin and adenosine does not affect the recognition of the substrate for this enzyme. However, this modification does effect the recognition of substrate by adenosine deaminase (32). In addition to chemical differences, there are stereochemical differences between formycin and adenosine at the glycosyl and the C(4')—C(5') bonds (33, 34). Our results show that any stereochemical differences between the 5'-triphosphates of the two nucleosides are not significant in adenylate cyclase activity.

Because FoTP is a fluorescent substrate for adenylate cyclase, we were able to observe cFoMP formation by fluorometry, using HPLC procedures similar to those previously used for separation of adenosine compounds (17). Studying the course of an enzymatic reaction with HPLC provides a number of advantages. For example, because of the relatively short retention times, it is possible to analyze the reaction mixture at least every 6 min and thereby follow the formation of reaction products almost continuously. As previously shown, HPLC can be used together with spectrophotometric detectors to follow the hydrolysis of ATP (17), with fluorescent analogs as substrates, the formation of reaction products such as cFoMP can be monitored by fluorometry, a change offering a number of advantages. First, as we showed in Fig. 3, fluorometry of cFoMP is more sensitive than spectrometry of CAMP and therefore this method is comparable in sensitivity to the $[^{32}P]$ATP-based assay for adenylate cyclase (35). Second, when fluorescence is monitored, interference from adenosine- or guanosine-containing compounds would not occur and therefore they need not be removed from the sample prior to injection. It might also be added that because the FoTP is produced synthetically, the problem of GTP contamination found with many ATP preparations can be avoided (35). Third, if a fluorometer is used in series with an absorbance detector (260 nm) then cAMP (or another adenosine compound) can be added to the sample as a recovery standard or as an internal marker to calibrate each column run and verify retention times. Finally, it should be pointed out that the method uses no radioactive compounds either in the assay or later as recovery standards. This latter fact should make fluorometry even more of an advantage not only for adenylate cyclase reactions but also for other ATP- or cAMP-dependent reactions, especially when the problem of radioactive waste disposal is considered (36).

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