Deoxyribonucleotide Biosynthesis in Green Algae. S Phase-Specific Thymidylate Kinase and Unspecific Nucleoside Diphosphate Kinase in *Scenedesmus obliquus*

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Dedicated to Professor Friedhelm Schneider on the occasion of his 60th birthday.

Algae, Cell Cycle, Deoxyribonucleotides, Nucleoside Diphosphate Kinase, *Scenedesmus obliquus*

NDP kinase and thymidylate kinase are essential for DNA precursor formation in that they phosphorylate the products of *de novo* deoxyribonucleotide biosynthesis, deoxyribonucleoside 5′-diphosphates and thymidine 5′-monophosphate to the corresponding triphosphates which then serve as DNA polymerase substrates. The two enzymes have been measured in synchronous cultures of the green algae, *S. obliquus*. Thymidylate kinase exhibits an activity peak at the 11–12th hour of the 24-hour cell cycle, coinciding with DNA synthesis. Enzyme activity is markedly stimulated in presence of fluorodeoxyuridine in the culture medium. This behaviour of dTMP kinase is very similar to that of three other S phase-specific peak enzymes previously analyzed in synchronous algae, *viz.* ribonucleotide reductase, thymidylate synthase, and dihydrofolate reductase. In contrast, NDP kinase exhibits high and constant activity through the entire cell cycle. The two kinases have been isolated from cell-free extracts, and separated from each other by chromatography on Blue Sepharose. The peak enzyme, dTMP kinase, has been purified to near homogeneity and its catalytic properties are described; the molecular weight is 56,000. NDP kinase activity is separable into two enzyme fractions, both of molecular weight 100,000 (or higher), which are unspecific with respect to ribonucleotide and deoxyribonucleotide substrates. Characterization and purification of the whole series of deoxyribonucleotide-synthesizing enzymes from one organism provides a basis for *in vitro* experiments towards reconstitution of an S phase-specific DNA precursor/DNA replication multienzyme aggregate.

Introduction

Passage of cells through the DNA synthesis (S) phase of their cell cycle requires a precisely scheduled and balanced supply of the four deoxyribonucleotides for DNA replication, which are not normally present in resting cells and tissues. The key enzymes of deoxyribonucleotide biosynthesis, ribonucleotide reductase and thymidylate synthase-dihydrofolate reductase have in fact pronounced S phase-specific activity maxima in synchronous animal, fungal, and plant cells [1–5]. These two enzyme systems, which materially limit cell cycle progress when inhibited [6] are apparently subject to common control mechanisms and tightly coupled in function and supramolecular organization [5, 7–9]. However, the immediate products of *de novo* deoxyribonucleotide synthesis are deoxyadenosine, deoxycytidine, and deoxyguanosine 5′-diphosphates, and thymidine 5′-monophosphate, respectively. Formation of the DNA polymerase substrates (deoxyribonucleoside 5′-triphosphates) from these nucleotides, as well as the incorporation of 2′-deoxyribonucleosides formed in salvage pathways, require additional kinase reactions, in particular nucleoside diphosphate (NDP) kinase and thymidylate kinase action.

It is not *a priori* possible to predict whether such auxiliary enzymes would also be cell cycle-dependent, and experimental evidence for an involvement of kinases in cell cycle and cell division control is only circumstantial. NDP kinase and thymidylate kinase

Abbreviations: dNMP, dNDP, dNTP, and NMP, NDP, NTP are the standard abbreviations for 2′-deoxyribonucleoside and ribonucleoside 5′-monophosphates, diphosphates, and triphosphates, respectively; FdU, 5-fluoro-2′-deoxyuridine; SDS, sodium dodecyl sulfate.

Enzymes: Lactate dehydrogenase (EC 1.1.1.27); dihydrofolate reductase (EC 1.5.1.3); ribonucleoside diphosphate reductase (EC 1.17.4.1); thymidylate synthase (EC 2.1.1.45); pyruvate kinase (EC 2.7.1.40); nucleoside diphosphate kinase (EC 2.7.4.6); thymidylate kinase (EC 2.7.4.9).

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are cell division cycle gene products in yeasts (*cdc22* in *Schizosaccharomyces pombe* [10], or *cdc8* in *Saccharomyces cerevisiae* [11], respectively). In bacterial and animal cells both kinases have been found associated with several other enzymes of DNA precursor and DNA synthesis [7–9, 12, 13]. Even less is known about their role in plants but steep increases in thymidylate kinase activity have been observed prior to DNA synthesis in synchronous cultures of *Chlorella* [14, 15] and at the beginning of the generative phase and cyst formation in *Acetabularia* [16].

We have previously analyzed the S phase-specific activity maxima of ribonucleotide reductase and thymidylate synthase in the unicellular green alga, *Scenedesmus obliquus* [4, 5], which is well suited for cell cycle studies. The virtually identical manifold overproduction of both enzymes in response to 5-fluoro-2′-deoxyuridine, an inhibitor of thymidylate synthesis has supported the concept of coordinate regulation of the "peak enzymes", and at the same time offered a more convenient source for purification and characterization of their molecular properties [17, 18]. Because it is not feasible to isolate an intact DNA precursor/DNA synthesis enzyme aggregate (if existent) from plant cells we attempt to reconstitute such a complex from the purified proteins. NDP kinase and thymidylate kinase have now been included in these studies. It was observed that the two enzymes, which are little known in green algae, behave very differently. A preliminary account has been given elsewhere [19].

Materials and Methods

Algal cultures

Axenic cultures of *Scenedesmus obliquus,* strain D3, were derived from stock cultures maintained in the collection of Dr. H. Senger, Fachbereich Biologie, Marburg. The algae were grown at 28 °C in a light thermostat (20,000 lx) in inorganic media as described [20], using 250 ml culture tubes aerated with 5 $l \cdot h^{-1}$ of an air/CO_2 (3%) mixture. Synchrony was induced in a periodic light/dark regime of 14/10 h, starting with a cell density of $4.4 \cdot 10^6$ cells $\cdot ml^{-1}$ at 0 h. Packed cell volumes were determined in a hematocrit tube by centrifuging (5 min at $1500 \times g$) 3 ml of a culture. FdU solutions were added to the cultures through sterile filters. Algae were harvested at the desired time by centrifugation in the cold (10 min at $1000 \times g$), resuspended in cold buffer, and centrifuged again. The cells could be stored frozen at −80 °C without loss in enzyme activities.

Chemicals and general methods

All chemicals and reagents were of highest purity available. Enzymes, coenzymes, and nucleotides were obtained from Boehringer Mannheim, and radioactive nucleotides from Amersham Buchler, Braunschweig. Chromatography media were from Whatman and Pharmacia. PEI cellulose thin layer plates and liquid scintillation fluid (Omniszintisol) were supplied by Merck, Darmstadt.

Protein was determined by the Lowry method [21], and DNA was measured fluorimetrically after reaction with 3,5-diaminobenzoic acid [22] as previously described [4]. SDS polyacrylamide gel electrophoresis was carried out on 12% gels in a standard buffer (pH 8.8) system [23]. Sucrose density gradients were produced in the SW 40 rotor of a Beckman L2-65B ultracentrifuge and collected with an ISCO 640 fractionator.

Enzyme purification

All preparative work was done at 0–5 °C. Packed algae (up to 10 ml) were suspended in 3 volumes of 0.05 M Tris-HCl buffer, pH 8.2 (measured at 5 °C) containing 10 mM mercaptoethanol, and homogenized for a total of 20 min in a refrigerated Bühler Vibrogen cell mill under addition of 0.7 mm glass beads. Smaller samples (up to 0.5 ml packed cells) were frozen in liquid nitrogen and ruptured with steel balls in a Micro-Dismembrator (Braun-Melsungen). The filtered homogenates were centrifuged at $30{,}000 \times g$ for 40 min. The clear green supernatant could be used for kinase activity determinations without further treatment.

The supernatant was made 1% in streptomycin sulfate by addition of a 5% solution. After 1 h the precipitate was removed by 30 min centrifugation, and the supernatant was dialyzed for 6 h against 0.02 M Tris-HCl buffer, pH 8.2, containing 10 mM mercaptoethanol. The solution was centrifuged again, if necessary, and was applied to a column (1.4×7.5 cm) of DEAE cellulose equilibrated in the same buffer (flow rate, 16 $ml \cdot h^{-1}$). After the removal of unbound proteins the column was eluted with a gradient of 0 to 0.4 M NaCl in buffer, and fractions of 2.5 ml were collected. The active frac-

tions were combined, and ammonium sulfate was added to 60% saturation. The resulting precipitate was collected by centrifugation, redissolved in the minimum volume of 0.05 M Tris-HCl buffer, pH 8.2, containing 10 mM mercaptoethanol, and dialyzed for several hours against buffer.

Gel permeation chromatography was done alternatively on columns (2.6 × 80 cm) of Sephacryl S400, equilibrated in 0.05 M Tris-HCl buffer, pH 7.8, containing 10 mM mercaptoethanol, or Sephadex G-100 equilibrated in the same buffer containing 0.1 M NaCl. The above enzyme solutions were chromatographed at a flow rate of 20 $ml \cdot h^{-1}$, and 10 ml fractions were collected. Active enzyme fractions were again precipitated at 60% ammonium sulfate saturation, and redissolved in and dialyzed against 0.01 M Tris-HCl buffer, pH 7.2, containing 2 mM mercaptoethanol for affinity chromatography.

Separation of NDP and dTMP kinase was achieved in analogy to a published procedure [24]. A column of Blue Sepharose CL-6B (1 × 16 cm) was washed with 0.5 M NaCl solution and equilibrated in the above 0.01 M Tris-HCl buffer. The column was charged with 3 ml of protein solution and eluted stepwise with 20 ml of equilibration buffer, 20 ml of 2 mM ATP/$MgCl_2$ in the same buffer, 40 ml of a Tris-HCl (pH 8.2, containing 10 mM mercaptoethanol) gradient increasing from 0.01 M to 1.5 M, and finally with 2.5 M Tris-HCl buffer, pH 8.2, containing 10 mM mercaptoethanol; flow rate, 7.7 $ml \cdot h^{-1}$. The thymidylate kinase fractions eluted last and had to be dialyzed against assay buffer prior to activity determination.

Enzyme assays

NDP kinase activity was determined at 25 °C by the spectrophotometric assay coupled to pyruvate kinase and lactate dehydrogenase (from rabbit muscle) [25]. Substrate concentrations were 0.7 mM CDP or dCDP, 2 mM ATP, 1.1 mM phosphoenolpyruvate, and 0.2 mM NADH; assay mixtures also contained 0.08 M Tris-acetate buffer, pH 7.6, 20 mM $MgCl_2$, and 70 mM KCl. NADH oxidation was recorded at 340 nm. Spectrophotometric NDP kinase activities were initially verified by the radiochemical method [26] using [2-^{14}C]CDP (spec. activity, 470 $mCi \cdot mmol^{-1}$) as substrate. Under the above conditions, 0.5 μCi [^{14}C]CDP plus 0.15 μmol unlabeled CDP were incubated with 20 μl protein extract in a total volume of 0.110 ml. After 10 min at 30 °C the reaction was terminated by boiling, precipitated protein was removed by centrifugation, and the nucleotides were separated on PEI cellulose sheets developed in methanol, 2 M Na-formate, and 4 M Na-formate solution. After drying, the radioactivity distribution among CTP, CDP, and CMP was determined in a thin layer scanner (BF model LB 2723). Both assay methods gave closely comparable results.

Thymidylate kinase activity was measured with [CH_3-^{3}H]dTMP (spec. activity, 42 $Ci \cdot mmol^{-1}$) as substrate [11, 15, 27]. Assays were run in duplicate; they contained, in a total volume of 0.10 ml Tris-HCl buffer (50 mM; pH 7.8), 1 μCi [^{3}H]dTMP plus 0.1 mM unlabeled dTMP, 10 mM ATP, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. After 30 min incubation at 30 °C the reaction was terminated by 3 min boiling, the precipitate removed by centrifugation, and the thymidine nucleotides separated by thin layer chromatography on PEI cellulose. Sheets were developed in 2 M acetic acid containing 0.5 M LiCl for 100 min. After drying, the radioactivity of dTTP (R_f = 0.05), dTDP (R_f = 0.4–0.5), and dTMP (R_f = 0.8) was determined by scanning, or by liquid scintillation counting of the excised nucleotide spots.

Results

Cell cycle dependence of NDP and thymidylate kinase activities

The analysis of deoxyribonucleotide-synthesizing enzymes in crude plant cell extracts is difficult because of the low activities and limited stability commonly encountered. Optimum culture, extraction, and assay conditions for ribonucleotide reductase and thymidylate synthase-dihydrofolate reductase, which are particularly critical proteins, in synchronous *S. obliquus* cells have been established previously [4, 5] and should be suitable for determination of the potentially less sensitive kinases, too. NDP kinase and thymidylate kinase were in fact readily measurable in cell-free algal extracts using radioactively labeled cytidine diphosphate (CDP) and thymidine monophosphate (dTMP) as substrates and direct chromatographic product estimation as described in the experimental section. Proper assay parameters such as incubation time, pH, substrate and ionic concentrations were confirmed at a later stage with purified enzyme fractions (see below).

The time course of the two kinase activities during a 24 h culture of *Scenedesmus obliquus* in a light-dark

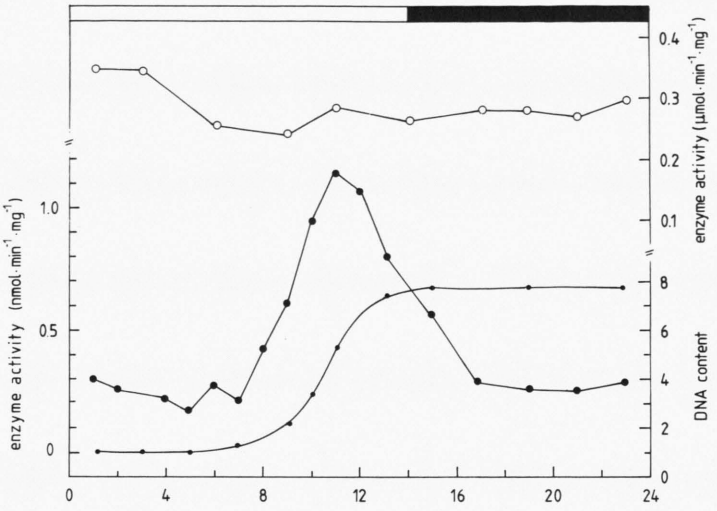

Fig. 1. Nucleotide kinase activities during the cell cycle of *Scenedesmus obliquus* cultured in a light/dark (14:10 h) regime. (•) DNA content per ml culture (relative figures); (●) thymidylate kinase activity (left scale); (○) NDP kinase activity (substrate CDP, right scale).

regime (14:10 h) is shown in Fig. 1. Thymidylate kinase is a peak enzyme with an activity maximum at around the 11th–12th hour whereas NDP kinase activity remains at a constant level during the entire cell cycle. The peak of dTMP kinase coincides with DNA synthesis (Fig. 1), with the appearance of a measurable dTTP pool [4], and with the activity maxima of ribonucleotide reductase and thymidylate synthase-dihydrofolate reductase [5]. Its specific activity is twofold higher than that of the dTMP synthase system. In contrast NDP kinase exhibits at least 100- to 1000-fold higher activity than the other enzymes of DNA precursor synthesis in cell-free extracts; as shown below, this high NDP phosphorylating activity represents several nucleoside diphosphate kinases.

Effect of fluorodeoxyuridine

The thymidylate synthase inhibitor, 5-fluoro-2′-deoxyuridine (FdU) does not affect thymidylate kinase *in vitro*. However, dTMP kinase activity increases up to threefold over the S phase activity peak when *S. obliquus* cells are cultured in the presence of FdU (Fig. 2). Maximum stimulation is observed when the nucleoside is added at a concentration of 20 mg/l (0.8×10^{-4} M) during the first hours of synchronous culture and growth is continued in the light for 24 h. This increase in enzyme activity is very similar to that described previously for ribonucleotide reductase and thymidylate synthase [5]. In contrast, NDP kinase activity in the algae is not influenced by fluorodeoxyuridine.

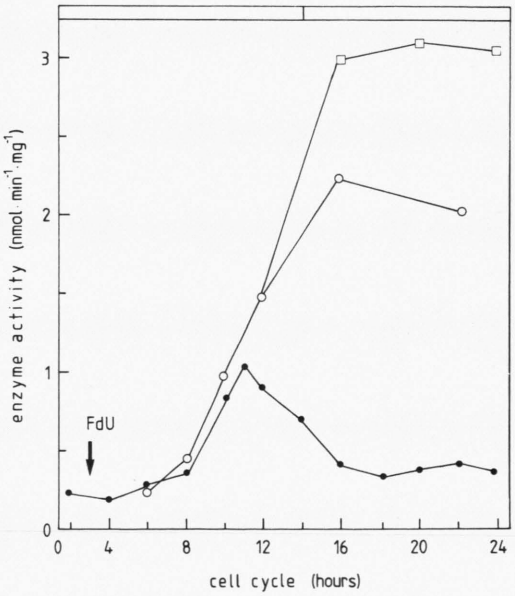

Fig. 2. Effect of fluorodeoxyuridine on thymidylate kinase activity in *S. obliquus*. FdU (20 mg/l culture) was added at the 3rd hour of the cell cycle (arrow). (●) No FdU (data from Fig. 1); (○) with FdU, light/dark regime; (□) with FdU, continuous light.

Purification of algal thymidylate kinase and NDP kinase

FdU-treated *Scenedesmus* cultures, which overproduce thymidylate kinase, were used as a source for enzyme purification. With a view on reconstitution of an S phase-specific DNA precursor-synthesizing enzyme aggregate [8] we have concentrated our efforts on the separation of both enzyme activities and on characterization of the peak enzyme, thymidylate kinase.

Ion exchange chromatography of algal protein extracts was found superior to ammonium sulfate fractionation as the initial step. Chromatography on DEAE cellulose in an NaCl gradient is depicted in Fig. 3. NDP kinase was clearly separated into two fractions, I and II, eluted at 0.10 and 0.22 M NaCl concentration, respectively. Thymidylate kinase cochromatographed with NDP kinase fraction I on these columns. Gel permeation chromatography on Sephacryl S400 (not shown) did not permit a reliable distinction of the various kinase fractions. Although NDP kinase occasionally chromatographed slightly before thymidylate kinase, NDP kinase I, II, and dTMP kinase all eluted in the M_r = 100,000 (± 20,000) size range of proteins.

Complete resolution of NDP kinase and thymidylate kinase was achieved by affinity chromatography on Blue Sepharose (Fig. 4). Whereas NDP kinase was completely desorbed from the column with 2 mM ATP at low ionic strength, the elution of dTMP kinase required high salt concentration (2.5 M Tris-HCl). The apparent fractionation of NDP kinase on Blue Sepharose indicated in Fig. 4 was not investigated further at this point. Following affinity chromatography, gel filtration of thymidylate kinase on a Sephadex G-100 column (Fig. 5) yielded a highly purified protein of molecular weight M_r = 56,000 which was used for the studies described below. Electrophoresis on denaturing SDS-polyacrylamide

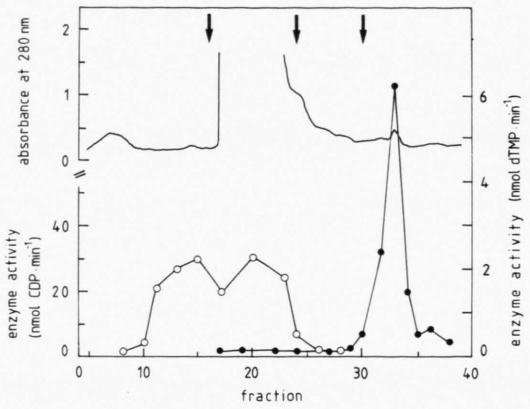

Fig. 4. Chromatography of *S. obliquus* nucleotide kinases on Blue Sepharose. The arrows on top indicate elution of the column with (from left) 1., 2 mM ATP-$MgCl_2$ in 0.01 M Tris-HCl buffer; 2., 0.01 M to 1.5 M Tris-HCl buffer; and 3., 2.5 M Tris-HCl buffer, pH 8.2. (●) Thymidylate kinase (right scale), (○) NDP kinase activity (left scale). The light absorption in fractions 17–24 represents ATP and not protein.

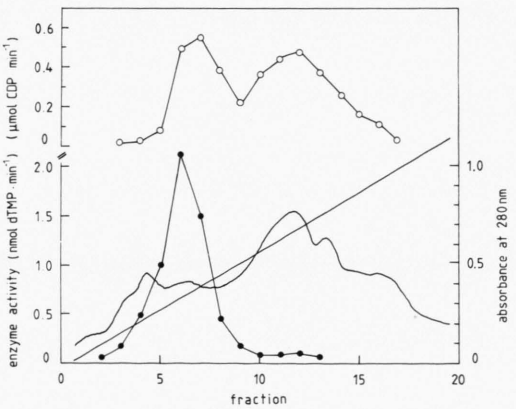

Fig. 3. Chromatography of *S. obliquus* protein extracts on DEAE cellulose. (●) Thymidylate kinase activity (lower left scale), (○) NDP kinase activity (upper scale). Drawn line: Protein absorption (right scale). The straight line represents an NaCl gradient from 0 to 0.4 M concentration.

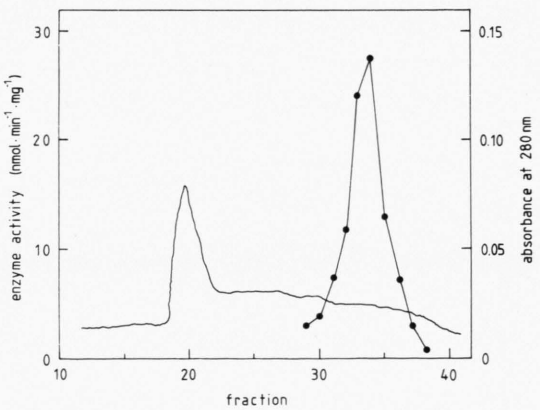

Fig. 5. Gel permeation chromatography of thymidylate kinase from *Scenedesmus obliquus* on Sephadex G-100. (●) Enzyme activity, (–) protein absorption).

gels exhibited only one strong protein band at M_r = 57,000 and a minor impurity at M_r = 63,000.

The specific activity of thymidylate kinase increased 300-fold during the entire purification. This must be considered a minimum value because at early purification stages dTMP kinase- and NDP kinase-catalyzed thymidylate phosphorylation (*i.e.*, formation of dTDP plus dTTP) cannot be safely differentiated.

Properties of thymidylate kinase

Some kinetic parameters of *S. obliquus* thymidylate kinase action *in vitro* were determined and the results are presented in Fig. 6–9. The enzyme possesses a broad pH optimum between pH 7 and 9 (Fig. 6). The apparent K_m value for thymidine 5′-monophosphate at 25 °C and pH 7.8 is 40 µM (Fig. 7). Magnesium ions in addition to ATP are essential for activity. Maximum rates were achieved at $\geqslant$ 5 mM concentration of the ATP:Mg^{2+} 1:1 complex, whereas an excess of free Mg^{2+} ions led to a marked reduction of activity. The reaction is highly specific for thymidylate as phosphate acceptor. Phosphorylation of deoxyuridine 5′-phosphate was so slow (at the best, 4% the rate of dTMP; data not shown) that a K_m value could not be determined, and phosphorylation of other deoxyribonucleotides was not detectable. However, the purified algal thymidylate kinase (freed from NDP kinase by affinity chromatography) does exhibit a certain thymidine diphosphate kinase activity. As shown in Fig. 9, a small amount of thymidine triphosphate accumulated during prolonged incubation, which was apparently formed from thymidine diphosphate. This side reaction does not disturb standard enzyme activity determinations.

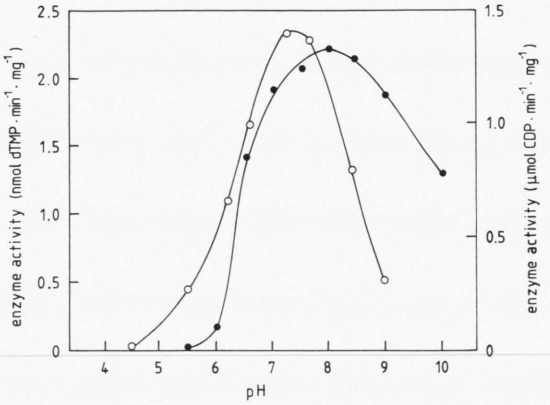

Fig. 6. Dependence of *S. obliquus* thymidylate kinase (●) and NDP kinase (○) activity on pH of the reaction medium.

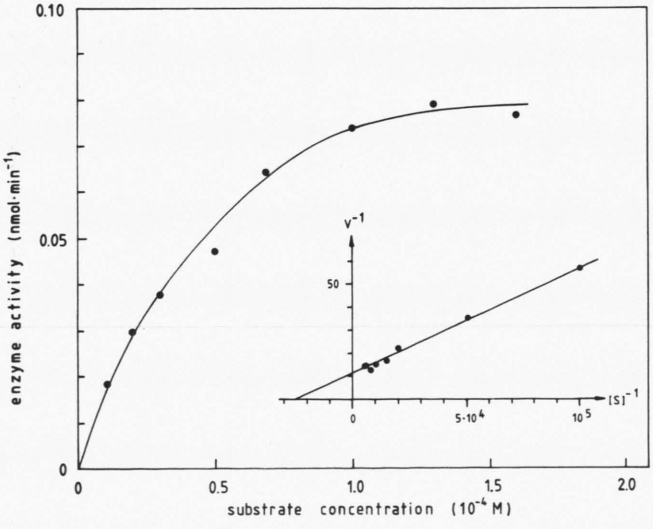

Fig. 7. Substrate (dTMP) saturation curve of *S. obliquus* thymidylate kinase. Insert: Double-reciprocal plot. Conditions: pH 7.8, 30 °C, 10 mM Mg-ATP.

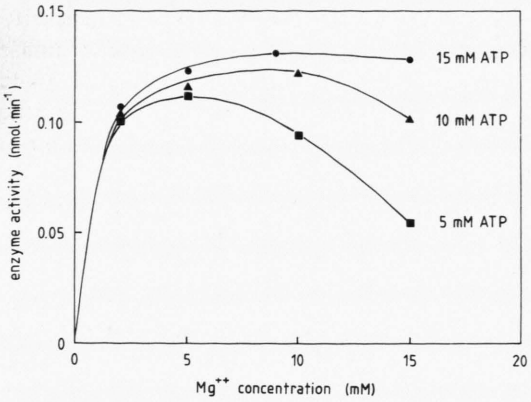

Fig. 8. Dependence of *S. obliquus* thymidylate kinase activity on Mg^{2+} ion and ATP concentrations. Conditions as in Fig. 7.

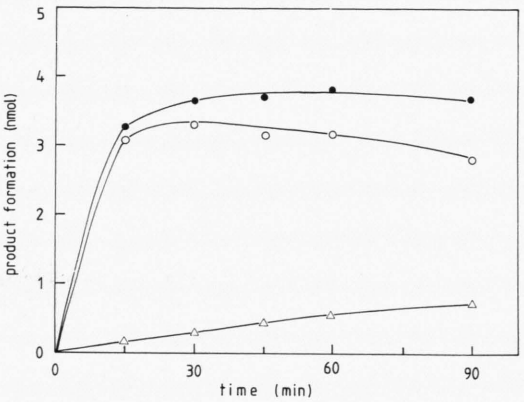

Fig. 9. Thymidylate kinase-catalyzed product formation from thymidine 5′-monophosphate. (○) Thymidine diphosphate; (△) thymidine triphosphate; (●) total products (dTDP + dTTP).

Partial characterization of NDP kinase

Complete purification of the NDP kinases present in *Scenedesmus obliquus* was not intended in this study because the activity was not cell cycle-dependent and was much higher than needed for cellular dNTP formation during DNA replication. The following characteristics were analyzed, however, for comparison with the other enzymes of dNTP biosynthesis.

Both NDP kinase fractions separated by DEAE cellulose chromatography (Fig. 3) were unspecific for ribonucleoside and deoxyribonucleoside 5′-diphosphate substrates. Because cellular threshold functions have been attributed to ribonucleotide reductase-catalyzed CDP reduction and intracellular dCTP levels [28] we compared CDP and dCDP phosphorylation by NDP kinase I and II quantitatively (Fig. 10). The two enzyme fractions differed very little in apparent K_m values (I: app. K_m = 0.2 mM; II: app. K_m = 0.3 mM for both CDP and dCDP) and only 2- to 3-fold in specific activities. The presence of thymidylate kinase in enzyme I may be neglected because dTMP kinase does not act on cytosine nucleotides. These small differences do not permit the assignment of physiological specificities.

The pH dependence of NDP kinase activity in *S. obliquus* extracts is included in Fig. 6; the optimum observed between pH 6.5 and 8 is comparable to that of other enzymes in algal nucleotide metabolism. A 10 mM magnesium ion concentration was required for maximum enzyme activity.

Finally the two NDP kinase fractions I and II were analyzed separately by sucrose density gradient centrifugation and gel permeation chromatography on Sephacryl S400 (not shown), and were found to behave almost identically in either method. The sedimentation and elution patterns indicated a molecular weight range of M_r = 100,000 to 160,000. More precise estimates could not be obtained, presumably because the enzyme fractions are still contaminated with other components of the deoxyribonucleotide-biosynthetic complex; NDP kinase activity is notorious for its association with other enzymes [8, 12, 13].

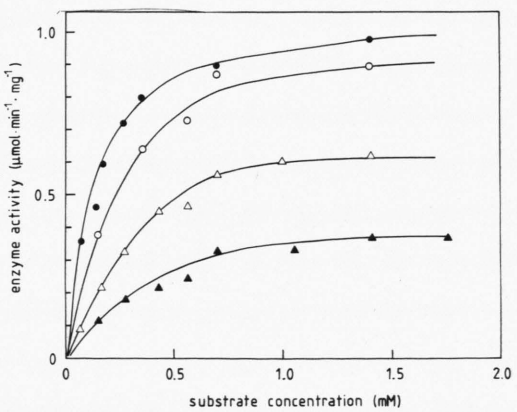

Fig. 10. Substrate concentration dependence of *S. obliquus* NDP kinases. (●) dCDP phosphorylation, enzyme fraction I; (○) CDP, enzyme I; (▲) dCDP phosphorylation, enzyme fraction II; (△) CDP, enzyme II. Spectrophotometric enzyme assays were run at pH 7.6 and 25 °C.

Discussion

Four different enzymes of DNA precursor biosynthesis have now been identified that exhibit activity peaks during the S phase of light/dark-synchronized cultures of *Scenedesmus obliquus, viz.* ribonucleotide reductase [4], thymidylate synthase [5], dihydrofolate reductase [18], and thymidylate kinase. We are not aware of any other such array of S phase-specific "peak enzymes". In addition, stepwise activity increases of dCMP deaminase [29] and DNA polymerase I (responsible for nuclear DNA synthesis) [30] have been observed in synchronous cultures of the related green algae, *Chlorella pyrenoidosa* and *C. ellipsoidea.* The functional advantage of these patterns for DNA replication is obvious. As deoxyribonucleotides have to be formed *de novo* in the extremely complex and easily inhibited ribonucleotide reductase reaction [31] a highly organized enzyme machinery for their efficient and rapid channeling towards DNA synthesis may in fact be a necessity. The identical effect of fluorodeoxyuridine, which leads to overproduction of the four enzymes, likewise indicates a common mode of regulation. It is still entirely unknown, however, which cellular signals trigger the coordinate induction of the proteins in G1 phase, and determine the cessation of enzyme synthesis after DNA replication is complete.

Thymidylate is unique among all nucleotides because it is the only end product of a specialized pathway and is utilized only in DNA. The cell cycle dependence of both thymidylate synthase and thymidylate kinase has not to our knowledge been followed under identical conditions in any one organism. The parallel peak profiles established in *S. obliquus* could support the earlier idea that cellular thymidylate pools, besides serving as DNA polymerase substrates, assume a role in the regulation of ribonucleotide reduction and DNA synthesis [32].

On the other hand nucleoside diphosphate kinase, which is also needed for the last step of dNTP formation and is a cell cycle gene product in yeast [10] does not fluctuate at all during the algal cell cycle (Fig. 1). Although the measured activity does not represent a homogeneous enzyme, the existence of a more specific, hidden dNDP kinase in the algae appears unlikely because all NDP kinase fractions were very active, but not very specific for ribo- and deoxyribonucleotide substrates (*cf.* Fig. 10). It is possible that the fairly high and constant NDP kinase activity, which is required in several reactions of nucleotide metabolism, makes a cell cycle-specific dNDP kinase unnecessary. Final conclusions, however, should await further purification and characterization of the algal enzymes, as well as analysis of NDP kinase in other lower eukaryotes.

Thymidylate kinase has not so far been purified extensively from plant sources. The properties of the highly purified algal enzyme described here are quite similar to those of most other dTMP kinases. Apparent K_m values for dTMP in the micromolar range, slightly alkaline pH optima, preference for an ATP:Mg^{2+} ratio of 1:1, and molecular weights in the range from 46,000 to 65,000 (*S. obliquus:* 56,000) have also been observed in the enzymes isolated from *E. coli,* avian, and mammalian cells [33–35].

Likewise, the NDP kinases present in *Scenedesmus* are not untypical in comparison with those from other sources. The occurrence of several, very similar proteins (isozymes) is well documented in mammalian tissues. They all bind to Blue Sepharose under closely comparable conditions, exhibit low substrate specificities, and apparent K_m values determined for CDP and dCDP are always in the millimolar range [36, 37]. Enzymes isolated from mammalian cells, yeast, and *Bacillus subtilis* possess molecular weights of about 100,000 [38–40]. This corresponds to the lower value found for the still impure algal enzyme preparations, and supports the argument that apparently heavier fractions may represent association with other proteins. Another plant NDP kinase, which has been characterized in pea seeds, exhibits a molecular weight of only 70,000 [41].

The individual properties of the two kinases characterized in this study do not *per se* provide new insight into the problem of an S phase-specific "replitase" [8] multienzyme aggregate of DNA precursor and DNA synthesis. However, the availability of several purified peak enzymes now permits to address the problem by *in vitro* reconstitution and kinetic coupling experiments.

Acknowledgements

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