Control of 6-(D-threo-1′,2′-dihydroxypropyl) pterin (dictyopterin) synthesis during aggregation of *Dictyostelium discoideum*

Involvement of the G-protein-linked signalling pathway in the regulation of GTP cyclohydrolase I activity

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6-(D-threo-1′,2′-Dihydroxypropylpterin (dictyopterin) has been identified in extracts of growing *Dictyostelium discoideum* cells [Klein, Thiery and Tatischeff (1990) Eur. J. Biochem. **187**, 665–669]. We demonstrate that it originates from GTP by *de novo* biosynthesis and that the first committed step is catalysed by GTP cyclohydrolase I, yielding dihydroneopterin triphosphate [neopterin is 6-(D-erythro-1′,2′,3′-trihydroxypropyl) pterin]. The GTP cyclohydrolase I activity is found in the cytosolic fraction and in a membrane-associated form. The level of a 0.9 kb mRNA coding for GTP cyclohydrolase I decreases to about 10 % of its initial value within 2 h after *Dictyostelium* cells start development induced by starvation. In the cytosolic fraction, the specific activities of GTP cyclohydrolase I, as well as the concentrations of (6R/S)-5,6,7,8-tetrahydrodictyopterin (H_4dictyopterin), follow this decline of the mRNA level. In the particulate fraction, however, the specific activities of GTP cyclohydrolase I and, in consequence, H_4dictyopterin synthesis, transiently increase and reach a maximum after 4–5 h of development. The time-course of H_4dictyopterin concentrations in the starvation medium closely correlates with its production in the membrane fraction. The activity of membrane-associated GTP cyclohydrolase I can be increased by pre-incubation of the cell lysate with guanosine 5′-[γ-thio]triphosphate and Mg^{2+}. This GTP analogue does not serve as a substrate and has no direct effect on the enzyme activity, indicating that a G-protein-linked signalling pathway is involved in the regulation of GTP cyclohydrolase I activity and thus in H_4dictyopterin production during early development of *D. discoideum*.

INTRODUCTION

Folate and (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4biopterin) are synthesized *de novo* from GTP [biopterin is 6-(L-erythro-1′,2′-dihydroxypropyl) pterin]. The first committed step in both pathways is catalysed by GTP cyclohydrolase I (EC 3.5.4.16) and results in the formation of dihydroneopterin triphosphate [neopterin is 6-(D-erythro-1′,2′,3′-trihydroxypropyl) pterin] [1]. It is generally accepted that in higher eukaryotes, H_4biopterin synthesis further proceeds through the subsequent action of 6-pyruvoyl-H_4pterin synthase (EC 4.6.1.10) [6-pyruvoyl-H_4pterin is 6-(1′,2′-dioxopropyl)-5,6,7,8-tetrahydropterin]. This enzyme eliminates the phosphate groups from dihydroneopterin triphosphate and catalyses an intramolecular reaction, yielding 6-pyruvoyl-H_4pterin. Sepiapterin reductase (EC 1.1.1.153) [sepiapterin is 6-(1′-oxo-2′-hydroxypropyl)-7,8-dihydropterin] is able to catalyse the reduction of each of the 1′- and 2′-oxo functions of this metastable intermediate to yield the final product H_4biopterin. The hydride equivalents are from NADPH. In addition, aldose reductase (EC 1.1.1.21) catalyses the reduction of the 2′-oxo group in 6-pyruvoyl-H_4pterin to yield 6-lactoyl-H_4pterin. This compound is then reduced by sepiapterin reductase. The involvement of aldose reductase is still controversial and measurement of its specific contribution to H_4biopterin synthesis in crude cell extracts has not been possible to date (for review see [2,3]).

In mammals, the biosynthesis of H_4biopterin is specifically regulated in different cell types through the activity of the rate-limiting enzyme GTP cyclohydrolase I. Cytokines, such as interferon γ (IFN-γ) and kit-ligand, were identified as primary regulators [4–6] that mediate the up-regulation of GTP cyclohydrolase I via increased levels of its steady-state mRNA [7]. The kit-ligand is also known as mast-cell growth factor, stem cell factor or steel factor. It binds to the product of the proto-oncogene c-kit, a receptor with tyrosine kinase activity [8]. For the activation of GTP cyclohydrolase I in murine mast cells by kit-ligand and for the co-stimulatory action of interleukin 2 and IFN-γ in T-cells, however, an additional regulation by post-translational modification has been suggested [9].

Dictyostelium cells grow as single amoebae. Starvation initiates a developmental programme. During its first phase the individual cells aggregate to form a multicellular mound. This is followed by the formation of a pseudoplasmodium and, eventually, a fruiting body that consists of dead stalk cells and viable spores. The aggregation process is coordinated by pulses of extracellular cyclic AMP (cAMP) which binds to cell-surface receptors and induces transient activation of several signal-transduction pathways. One of these causes the activation of adenylate cyclase (EC 4.6.1.1), inducing further synthesis and secretion of cAMP into the extracellular medium in the 'relay response' (for review see [10–13]). This signalling pathway is linked to heterotrimeric G-proteins and involves the exchange of GTP for GDP on the

Abbreviations used: H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; biopterin, 6-(L-erythro-1′,2′-dihydroxypropyl) pterin; H_4dictyopterin. (6*R*/*S*)-5,6,7,8-tetrahydrodictyopterin; dictyopterin, 6-(D-threo-1′,2′-dihydroxypropyl) pterin; neopterin, 6-(D-erythro-1′,2′,3′-trihydroxypropyl) pterin; 6-pyruvoyl-H_4pterin, 6-(1′,2′-dioxopropyl)-5,6,7,8-tetrahydropterin; sepiapterin, 6-(1′-oxo-2′-hydroxypropyl)-7,8-dihydropterin; monapterin, 6-(L-threo-1′,2′,3′-trihydroxypropyl) pterin; GTP[γS] and ATP[γS], guanosine and adenosine 5′-[γ-thio]triphosphates; cAMP, cyclic AMP; IFN-γ, interferon γ; PB, development buffer KH_2PO_4/Na_2HPO_4 (17 mM, pH 6.0).

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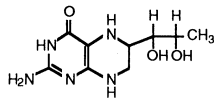
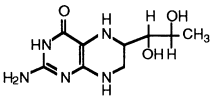


Tetrahydrobiopterin

6-(L-erythro-1',2'-dihydroxypropyl)-5,6,7,8-tetrahydropterin



Tetrahydrodictyopterin

6-(D-threo-1',2'-dihydroxypropyl)-5,6,7,8-tetrahydropterin

Figure 1 Structure of H_4biopterin and H_4dictyopterin

α-subunit and the dissociation of the α-subunit from the $\beta\gamma$-subunits [10–13].

For growth of *D. discoideum* amoebae, folate is an essential vitamin [14]. Secreted by bacteria, it also serves as a chemoattractant and acts to direct the amoebae to their bacterial prey [15]. The active structural element appears to be the pterin moiety [16,17]. Other functions of folates and unconjugated pteridines in the metabolism of *Dictyostelidae* have been proposed but are not yet clearly established. In *Dictyostelium lacteum*, a pterin derivative, rather than cAMP, was reported to mediate cell aggregation during the first developmental stage. A number of (6)-polyhydroxyalkyl pterins were also active but the structure of the natural compound remained unclear [18]. It has recently been proposed that in *D. discoideum*, the G-protein subunit $G\alpha_4$, which is essential for controlling prespore cell fate, couples to receptors specific for folate or related, yet unidentified, pterins [19].

De novo H_4biopterin synthesis from GTP has been demonstrated in the acellular slime mold *Physarum polycephalum* [20], which is also able to synthesize folate from this nucleotide [20]. In cellular slime molds which are grouped with the order of *Rhizopoda* [21], isoxanthopterin [22] and lumazines [22,23] found in the culture medium of *D. discoideum* were considered as folate decomposition products arising through the action of deaminase(s) [23]. These deaminases, which use folic acid as well as pteridines as substrates, occur in several *Dictyostelium* species as extracellular and membrane-bound forms, yielding 2-deamino folate and lumazines respectively [24,25]. The activity of the deaminase(s) increases during the aggregation phase, and addition of cAMP or folate enhances deaminase production ([26]; for review see [27]). More recently, a pteridine from cell extracts of *D. discoideum* was identified as 6-(D-threo-1,2-dihydroxypropylpterin) (see Figure 1). It was named dictyopterin and represents a new naturally occurring isomer of L-biopterin [28]. It was also shown that dictyopterin is deaminated by an extracellular pterin deaminase to D-threo-1,2-dihydroxypropyl lumazine (D-dictyolumazine) [29].

In this study we have followed the production of dictyopterin during the aggregation of *D. discoideum*. We demonstrate that it originates by *de novo* synthesis from GTP and that a G-protein-linked signalling pathway is involved in the regulation of GTP cyclohydrolase I activity during the course of cell aggregation.

EXPERIMENTAL

Materials

Yeast extract and neutralized peptone for cell culture was from Oxoid (Wesel, Germany). Nucleopore filters were from Costar (Tübingen, Germany) and filter holders from Sartorius (Göttingen, Germany). Guanosine and adenosine 5′-[γ-thio]triphosphates (GTP[γS] and ATP[γS] respectively) and cAMP were from Sigma (Deisenhofen, Germany), alkaline phosphatase (high concentration) from Boehringer (Mannheim, Germany) and pterins from Dr. B. Schircks Laboratories (Jona, Switzerland). The sources of the chemicals that were used for determination of enzymic acitvities, for protein determination and for analysis of pterins by HPLC were as described previously [30,31]. Details of the materials that were used for Northern blot analysis are given in [7,32].

Culture conditions and membrane preparation

D. discoideum strain Ax2 was grown axenically under agitation (150 rev./min) at 22 °C in the medium described in [33] until the cells reached a density of $(3–6) \times 10^6$ cells ml^{-1}. To start development, the cells were washed twice, resuspended in KH_2PO_4/Na_2HPO_4 (17 mM, pH 6.0) (PB) at a density of 1×10^7 cells ml^{-1} and agitated (150 rev./min) at 22 °C [34]. Cells which were subsequently used for Northern blot analysis were alternatively developed on a solid surface as described in [35]. Both methods yielded the same results. Cells were taken at the times indicated and washed twice in cold PB. Aliquots of $(4–5) \times 10^7$ cells were resuspended in 350 μl of 40 mM Hepes/NaOH buffer (pH 7.0)/0.5 mM EDTA and lysed by elution through a nucleopore polycarbonate filter (pore size 3 μm) as described in [34]. The lysate was centrifuged for 5 min at 12000 ***g*** and the pellet was resuspended as described below. During isolation, the samples were kept at 4 °C.

Determination of pterins and of enzymic activities

The pelleted membrane fraction was washed in 2 ml of 40 mM Hepes/NaOH buffer (pH 7.0)/40 mM EDTA and resuspended in 200 μl of 40 mM Hepes/NaOH buffer (pH 7.7)/2.5 mM EDTA. Dictyopterin was determined in 30–50 μl aliquots of this membrane fraction and in the supernatant obtained by centrifugation of the Nucleopore lysate. The samples were acidified, oxidized by iodine, deproteinized by trichloroacetic acid, prepurified by cation-exchange chromatography and separated by reverse-phase HPLC with fluorometric detection. Details of this method as well as the discrimination of aromatic, di- and tetra-hydro forms of the pterin have been described previously [30]. Besides the solvent systems described in [36], a modified system (30 mM ammonium formate/30 mM formic acid/3 % methanol) was used to optimize the separation of dictyopterin. For determination of released H_4dictyopterin in the PB starvation medium, 100 μl of 1 M HCl was added to 500 μl aliquots, oxidized by iodine and processed as described above.

The activity of GTP cyclohydrolase I in the washed membranes and in the supernatant fraction was determined after oxidation of the reaction product to neopterin triphosphate [30]. The assay was performed in 50 mM Tris/HCl buffer (pH 8)/2.5 mM EDTA, containing 500 μM GTP and protein in a total volume of 65 μl. After incubation (60 min, 37 °C) the reaction products were oxidized by iodine at pH 1. The neopterin phosphates were measured directly after ion-pairing HPLC with the solvent system described in [36]. Adjustment of this solvent system to pH 7.0 was performed by acetic acid instead of H_3PO_4. In addition, neopterin was measured after adjusting 10 μl of the oxidized reaction product to pH 8.8 using 1.5 M Tris/HCl buffer and digestion with alkaline phosphatase (2 units, 37 °C, 60 min). For HPLC separation of neopterin, the modified solvent system described above was used. The sepiapterin reductase assay was performed in 50 mM Tris/HCl buffer (pH 8) and contained 40 μM sepiapterin, 100 μM NADPH and protein in a total

volume of 100 μl [30]. After incubation (60 min, 37 °C) and iodine oxidation of the reaction product dihydrobiopterin, biopterin was determined by reverse-phase HPLC with the modified solvent system described above. The activity of 6-pyruvoyl-H_4pterin synthase was measured by formation of H_4biopterin from dihydroneopterin triphosphate. This substrate was freshly prepared from GTP (500 μM) and GTP cyclohydrolase I, obtained as a recombinant fusion protein [37]. It was incubated in 50 mM Tris/HCl buffer (pH 7.8)/2.5 mM EDTA for 40 min at 37 °C. EDTA was then quenched by Mg^{2+} (final concentration 10 mM). The membrane fraction or the supernatant fraction of the Nucleopore lysate (30–50 μl aliquots) respectively were added together with excess purified sepiapterin reductase [38] and NADPH (20 μM) and incubated for 60 min at 37 °C. The resulting H_4biopterin was oxidized to biopterin by iodine, separated by reverse-phase HPLC with the modified solvent system described above and fluorimetrically determined. The values for 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase are means of triplicate determinations which varied by $< 10\%$. Protein was estimated by the Coomassie Blue dye binding reagent (Bio-Rad Assay Reagent) according to the manufacturer's instruction.

Activation of GTP cyclohydrolase I *in vitro*

The activation assays *in vitro* were performed with membranes and supernatant fractions, the preparation of which is described above. For GTP activation of G-proteins, lysis was performed in the presence of 20 μM GTP[γS] with 5 mM Mg^{2+} [34]. For cAMP-mediated stimulation, 20 μM ATP[γS] and 5 mM Mg^{2+} were present in the lysis medium. cAMP (10 μM) was immediately added to the lysate and incubated for 5 min at 20 °C to allow nucleoside-diphosphate kinase reactions [39]. Subsequently, the membrane fraction and the supernatant were separated at 0 °C as described above. The membrane fraction was resuspended in 200 μl of cold Hepes/NaOH buffer and in both fractions Mg^{2+} was quenched by EDTA (10 mM final concentrations of EDTA in both plasma and membrane fraction). They were assayed for GTP cyclohydrolase I activity as described above.

Northern blot analysis for GTP cyclohydrolase I mRNA expression

A GTP cyclohydrolase I 284 bp cDNA probe was generated by reverse transcriptase PCR. The RNA was isolated from vegetatively growing *Dictyostelium* cells by a modified version [32] of the procedure of Chirgwin [40]. Primers were used that matched the cDNA in regions of high sequence identity [41]. The sequences of the primers are: 5′-AYGARGAYCAYGAYGARATGG-3′ and 5′-ACCNCGCATNACCATRCAC-3′.

The specificity of the probe was confirmed by direct sequencing. For Northern blot analysis two strategies were used. In the first method, cells were lysed in guanidinium isothiocyanate and total RNA was isolated by caesium chloride gradient centrifugation. RNA aliquots (20 μg) were size-fractionated on a denturating formaldehyde/Mops gel and transferred to Nylon membranes by vacuum blotting. Probes were labelled by the PCR method starting from 1 ng of the purified plasmid DNA. Details of the methods, as well as prehybridization and hybridization, are described in [32]. The membranes were finally washed at 42 °C with 0.1 × SSC (15 mM sodium chloride/1.5 mM trisodium citrate, pH 7)/0.1 % SDS. Quantitative data were obtained by exposing the blots to the Fujix BAS 1000 Phospho Imager. In the alternative method cells were lysed by Tris buffer with nucleotide–vanadyl complex/SDS and phenol/chloroform. RNA was precipitated with ethanol and 20 μg aliquots were size fractionated in a Mops gel. Details of the method are described in [42]. Random probe labelling of the purified *Aat*II–*Sac*I fragment was performed according to the Amersham RPN 1600 Z procedure. Transfer on Nylon membranes, prehybridization and hybridization are described in [42]. Membranes were finally washed in 2 × SSC (300 mM sodium chloride/30 mM trisodium citrate, pH 7.0)/1 % SDS. For quantitative evaluation, the autoradiograms were densitometrically scanned. Both strategies yielded identical results.

RESULTS

Identification of *Dictyostelium* pterins

Figure 2 shows the identification of *Dictyostelium* pterins in the starvation medium (A), in the membrane (B) and in the plasma (C) fractions. Dictyopterin is the predominating pterin and differential oxidation in acidic and alkaline iodine indicates that it is present as its tetrahydro form. It comprises 97 % of total pterins in the medium, 94 % in the membranes and 67 % in the plasma. Among the minor pteridine fractions, biopterin and 6-hydroxymethylpterin were identified. In the plasma fraction about 15 % of total pterin was found as biopterin.

Northern blot analysis of GTP cyclohydrolase I mRNA

Northern blot analysis was performed by two alternative strategies (see the Experimental section) which yielded identical results. Growing cells show substantial expression of a 0.9 kb message specific for GTP cyclohydrolase I. Its level declines within 2 h of development to about 15 % of initial values (Figure 3A) and does not recover up to the end of the aggregation period

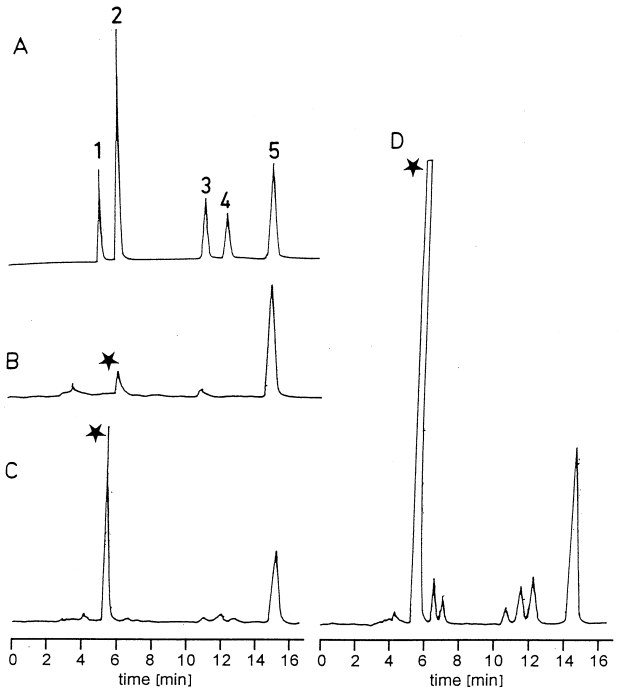


Figure 2 HPLC profile of pteridines in cell extracts of *D. discoideum*

The extracts were oxidized by acidic iodine and pre-purified by cation-exchange chromatography as described in the Experimental section. Solvent: 30 mM ammonium formate/30 mM formic acid/3% methanol. (**A**) Standard pterins: 1 = neopterin, 2 = 6-(L-threo-1′,2′,3′-trihydroxypropylpterin) (L-monapterin), 3 = biopterin, 4 = 6-hydroxymethylpterins, 5 = dictyopterin; (**B**) starvation medium; (**C**) membrane fraction; (**D**) plasma fraction. ★, L-monapterin, which was added as standard before the oxidation step.

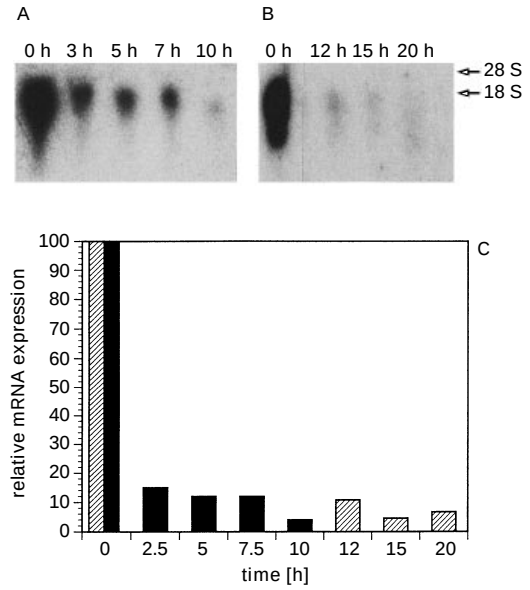


Figure 3 Northern blot analysis of GTP cyclohydrolase I mRNA expression during the development of *Dictyostelium* cells

(**A**) Comparison of growing cells and cells after 10 h of starvation. (**B**) Comparison of growing cells and cells developed for between 12 and 20 h. (**C**) Quantitative estimation of the signals: ■, results from experiment (**A**); ▨, results from experiment (**B**). Cells were developed in liquid suspension in buffer (**A**) or on a solid surface (**B**). Data points for development between 2 and 10 h were overlapping in both experiments and showed identical results.

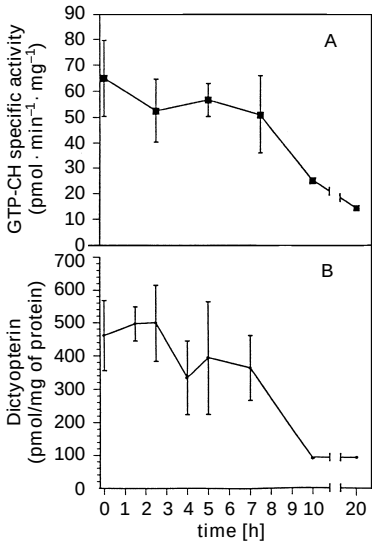


Figure 4 Time-course of GTP cyclohydrolase I activity (A) and H_4dictyopterin levels (B) in the cytosolic fraction during *Dictyostelium* development

H_4dictyopterin was determined as dictyopterin after acidic oxidation and HPLC separation, as described in the Experimental section (±S.D): $n = 6$–8 for GTP cyclohydrolase I activity; $n = 3$–5 for H_4 dictyopterin levels. Values at 10 and 20 h of development are obtained from unfractionated cell extracts and are means of duplicate determinations.

(Figure 3B). The decline in GTP cyclohydrolase I mRNA expression was the same whether the cells were developed in suspension culture or on a solid surface.

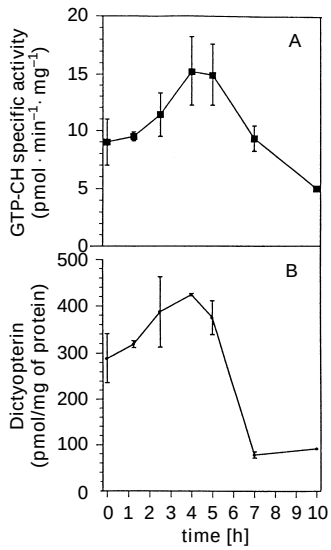


Figure 5 Time-course of GTP cyclohydrolase I activity (A) and H_4dictyopterin levels (B) in the membrane fraction during *Dictyostelium* development

H_4dictyopterin was determined as dictyopterin after acidic oxidation and HPLC separation, as described in the Experimental section (±S.D.): $n = 4$–6 for GTP cyclohydrolase I activity and for H_4dictyopterin levels.

H_4dictyopterin levels and activity of GTP cyclohydrolase I

The time-courses of H_4dictyopterin levels and of specific GTP cyclohydrolase I activities were determined separately in the supernatant and in the washed membrane fractions after rapid lysis of the cells through Nucleopore membranes [34]. In the cytosolic fraction, H_4dictyopterin levels and the specific activity of GTP cyclohydrolase I gradually decrease during the first 8 h of development. This is followed by a steep decline at the time of formation of the aggregates. After 20 h almost no H_4dictyopterin synthesis can be detected (Figures 4A and 4B). The minor pterin fractions, e.g. H_4biopterin or 6-hydroxymethylpterin, decrease in parallel with dictyopterin levels (results not shown).

The specific activity of GTP cyclohydrolase I and of H_4dictyopterin production in the cytosolic fraction (Figures 4A and 4B) is calculated on the basis of protein concentration. In order to estimate the dictyopterin production per cell, the protein content during the aggregation process has to be taken into account. Under our experimental conditions the total protein content remains constant for up to 6 h, then decreases to about 85% after 8 h and to about 50% after 20 h. Therefore, GTP cyclohydrolase I activity and H_4dictyopterin production per cell almost completely disappear at late stages of the aggregation period. Comparison with the Northern blot analysis (Figure 3) shows that the decline in the enzymic activity follows the decline of steady-state GTP cyclohydrolase I mRNA expression, with a delay period of 4–5 h.

In contrast to the cytosolic fraction, the specific activity of GTP cyclohydrolase I and the H_4dictyopterin levels transiently increase in the membrane fraction during the aggregation period, with a maximum after 4–5 h (Figures 5A and 5B). At this time about 10% of total cellular GTP cyclohydrolase I activity and 25–30% of total H_4dictyopterin are associated with the membrane fraction.

The time-course of H_4dictyopterin production in the membrane fraction closely correlates with its concentration in the

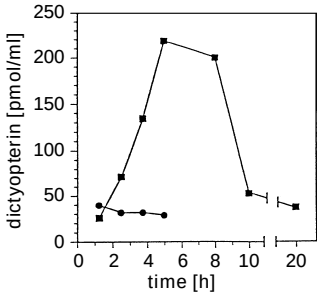


Figure 6 Time-course of H_4dictyopterin accumulation in the extracellular medium during *Dictyostelium* development

■, Control cells; ●, cells cultivated with 3.3 mM 2,4-diamino-6-hydroxypyrimidine. H_4dictyopterin was determined as dictyopterin after acidic oxidation of the cultivation medium and HPLC separation, as described in the Experimental section. Values are means of triplicate determinations.

Table 1 *In vitro* activation of GTP cyclohydrolase I in *Dictyostelium* membranes

The data represent the means ± S.D. (*n* in parentheses) of the experiments. The specific activity of control cells was 10.8 ± 3.7 pmol · min^{-1} · mg^{-1} and was set as 100%. For direct activation by GTP[γS], lysates were prepared in the presence of 20 mM GTP[γS] and 5 μM Mg^{2+}. For activation by ATP[γS]-derived GTP[γS], cells were lysed in the presence of 20 μM ATP[γS] and 5 mM Mg^{2+}, then 20 μM cAMP was added to the lysate. Membranes were separated and after quenching of Mg^{2+} by EDTA they were assayed for GTP cyclohydrolase I activity. *$P = < 0.05$.

Additions to the lysis buffer	Additions to the lysate	% of control
GTP[γS], Mg^{2+}	–	210.0 ± 0.0* (7)
GTP[γS], no Mg^{2+}	–	95.0 ± 5.1 (3)
ATP[γS], Mg^{2+}	cAMP	177.1 ± 39.1* (5)
ATP[γS], Mg^{2+}	–	113.6 ± 8.9 (3)
–	cAMP	100.7 ± 27.1 (3)

starvation medium. The pterin rapidly accumulates in the extracellular medium and reaches highest concentrations after 4–5 h (Figure 6). The subsequent rapid decline suggests that active pterin catabolism takes place. Inhibition of cellular GTP cyclohydrolase I activity by 2,4-diamino-6-hydroxypyrimidine [2,43] almost completely abolishes the accumulation of H_4dictyopterin in the culture medium (Figure 6), demonstrating that the released H_4dictyopterin originates from *de novo* synthesis during the process of differentiation.

Activation of GTP cyclohydrolase I *in vitro*

The increase of GTP cyclohydrolase I activity in the membrane fraction during aggregation is in contrast to its decrease in the the cytosolic fraction. In order to test whether a cAMP-dependent signalling pathway and heterotrimeric G-proteins are involved in the activation of GTP cyclohydrolase I, we have prepared membranes under conditions that preserve the potential interaction between the cAMP receptor and its signal-transducing proteins. Activation of G-proteins was achieved by lysis of the cells in the presence of GTP[γS] and rapid separation of the membranes. Table 1 demonstrates that activation of G-proteins *in vitro* results in at least 100% increase of GTP cyclohydrolase I activity. No activation occurs when Mg^{2+} is omitted during lysis.

Membrane-associated nucleoside-diphosphate kinase (EC 2.7.4.6) in *Dictyostelium* has been shown previously to be stimulated by cell-surface cAMP receptors [39]. This stimulation subsequently increases the levels of GTP. G-protein activation can therefore also be achieved *in vitro* by cAMP and ATP[γS]. The stimulated nucleoside-diphosphate kinase then produces GTP[γS]. Table 1 demonstrates that this pathway also results in activation of GTP cyclohydrolase I. Unstimulated nucleoside-diphosphate kinase was apparently not capable of generating sufficient GTP[γS] for the activation of GTP cyclohydrolase I. The endogenous GDP pool was shown not to be a limiting factor in the kinase reaction [39]. In our experiments, additional GDP (50 μM) reduced the subsequent production of dihydroneopterin triphosphate and was therefore omitted. This observation is in accord with the reported inhibitory effect of GDP on GTP cyclohydrolase I [44].

GTP[γS] increased the activity of GTP cyclohydrolase I only in the presence of Mg^{2+}. This Mg^{2+} in the lysate had to be quenched by EDTA before assaying the activity of the enzyme under substrate-saturating conditions (220 μM GTP). In separate experiments we examined whether GTP[γS] itself could serve as substrate or as a positive effector for GTP cyclohydrolase I to yield additional dihydroneopterin triphosphate. Incubation of *Dictyostelium* cell extracts with GTP[γS] (230 μM), however, resulted in no detectable formation of dihydroneopterin phosphates. Moreover, addition of GTP[γS] (115 μM) to the cytoplasmic fraction, to the membrane fraction after lysis or to the assay medium had no effect on the production of dihydroneopterin triphosphate from GTP (115 μM) by GTP cyclohydrolase I. In three experiments, addition of GTP[γS] did not enhance the activity of GTP cyclohydrolase I (results not shown). Thus, it neither serves as a substrate nor does it function as as a positive effector through direct interaction with the enzyme.

Activity of 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase

The activities of both 6-pyruvoyl-H_4pterin synthase and sepiapterin reductase were also analysed and compared with those of GTP cyclohydrolase I. In extracts of growing *Dictyostelium* cells the activity of 6-pyruvoyl-H_4pterin synthase amounts to 163 pmol · min^{-1} · mg^{-1} and thus exceeds the activity of GTP cyclohydrolase I (see above) about 3-fold. The reduction of sepiapterin yields 60.7 pmol · min^{-1} · mg^{-1} H_4biopterin. The specific activities of both enzymes increase during development to 305 and 122 pmol · min^{-1} · mg^{-1} respectively after 8 h. This apparent increase, however, proceeds during a period when cellular protein levels decrease by approx. 50% (see above). Thus, on a cellular basis the activities of both enzymes do not significantly change during development. These constant levels are in contrast to GTP cyclohydrolase I. They further point to the view that H_4dictyopterin synthesis during the aggregation period of *Dictyostelium* cells is controlled via the initial enzyme GTP cyclohydrolase I.

DISCUSSION

Little is known about pteridine metabolism in *D. discoideum*, and reports about the origin of unconjugated pterins are controversial. In this paper we have identified a regulatory reaction during starvation-induced development. First of all, we have shown that the unconjugated pterins in *Dictyostelium* are not degradation products of folate, which this organism must acquire from the external medium. As in mammals, unconjugated pterins

in *Dictyostelium* originate from GTP by *de novo* biosynthesis upon the action of GTP cyclohydrolase I.

Our study reveals different time-courses of GTP cyclohydrolase I activity in the membrane and cytosolic fractions. When the cells start development in suspension culture or on a solid surface, the levels of their GTP cyclohydrolase I mRNA decreases within 2 h to about 10 % of initial levels. The specific activity of the enzyme in the supernatant fraction follows this decline and suggests a half-life of the protein of 2–3 h. In contrast, the fraction of enzyme activity that remains associated with the membrane, transiently increases during aggregation of the cells, with a maximum at 4–5 h of development. Interestingly, the number of the cAR1 cAMP receptors and the amount of the G-protein subunit $G\alpha_2$ reaches a maximum at this period (for review see [13]). The subsequent steep decline in H_4dictyopterin levels in the starvation medium appears to be caused by degradation, which is likely to result from the production of membrane-bound and extracellular pterin deaminases. Their activity increases during the first hours of development and they are induced by cAMP [24–26].

Our experiments indicate that the production of H_4dictyopterin is due to increased activity of membrane-associated GTP cyclohydrolase I. The time-courses of H_4dictyopterin release and of the activity of membrane-associated GTP cyclohydrolase I are closely correlated. Moreover, in both the starvation medium and the membrane fraction, H_4dictyopterin dominates. It represents 94 and 97 % of total pterins respectively, and only trace amounts of H_4biopterin are found; only in the cytosolic fraction are substantial amounts of H_4biopterin also accumulated.

Finally, it is shown for the first time that the up-regulation of GTP cyclohydrolase I activity in the membrane fraction is a post-translational event. *In vitro*, this activation can either be achieved by direct addition of the non-hydrolysable analogue, GTP[γS], or as a consequence of GTP[γS] synthesis from ATP[γS] by nucleoside-diphosphate kinase [39], an enzyme which exchanges phosphates (and thiophosphates) between tri- and di-nucleotides. GTP[γS], together with Mg^{2+}, has to be present during lysis of the cells, and it serves neither as a substrate nor as a positive effector acting directly on the enzyme. This indicates the involvement of the G-protein-linked signalling pathway in the activation of GTP cyclohydrolase I and may explain the observation that in the membrane fraction the activity of GTP cyclohydrolase I increases during the aggregation period of *D. discoideum*. This entails increased release of H_4dictyopterin despite rapidly decreasing levels of GTP cyclohydrolayse I in the cytosolic fraction. In this model system the reaction mechanisms involved have to be identified further. Given the analogy between the *Dictyostelium* and mammalian G-protein signalling pathways [12,13] the results point to a possible similar role of G-proteins in the control of GTP cyclohydrolase I activity in mammalian cells.

The present study shows that H_4dictyopterin originates from GTP and our work focussed on the control of its biosynthesis by GTP cyclohydrolase I. It is well established that in mammalian cells, the subsequent step in H_4biopterin synthesis is catalysed by 6-pyruvoyl-H_4pterin synthase (for review see [2,3]). In *Dictyostelium*, its specific activity markedly exceeds that of GTP cyclohydrolase I and remains at constant levels during the whole aggregation phase. This confirms that H_4dictyopterin synthesis is controlled by GTP cyclohydrolase I. Moreover, the high activity of 6-pyruvoyl-H_4pterin synthase suggests that 6-pyruvoyl-H_4pterin takes part in H_4dictyopterin synthesis. These data suggest that the pathway leading to formation of either the L-erythro or the D-threo isomer most likely branches from the intermediate 6-pyruvoyl-H_4pterin (which has no stereocentres in the side-chain) and occurs during its reduction. It was outlined in the beginning that in the case of H_4biopterin synthesis the mechanisms of this reaction are not yet fully understood (see the Introduction). Moreover, the reactions which lead to the formation of H_4dictyopterin from 6-pyruvoyl-H_4pterin remain to be elucidated. For the formation of H_4dictyopterin, a final epimerization of primarily synthesized H_4biopterin has to be considered, which is analogous to the formation of L-threo-dihydroneopterin triphosphate from D-erythro-dihydroneopterin triphosphate in *Escherichia coli* [45]. An analogue epimerase activity, however, could not be detected in *Dictyostelium* extracts (results not shown). This additionally directs further analysis of H_4dictyopterin synthesis primarily to the reactions which catalyse the reduction of 6-pyruvoyl-H_4pterin.

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