A 138-kDa Glycoprotein from Dictyostelium Membranes with Folate Deaminase and Folate Binding Activity*

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A 138-kDa glycoprotein comprising folate deaminase activity was purified to apparent homogeneity from membranes of Dictyostelium discoideum. Deaminase activity could be effectively inhibited by p-chloromercuriphenylsulfonate. This treatment protected folate from deamination and thus allowed investigation of folate binding to deaminase fractions. Two types of folate binding sites, differing in affinity and specificity, were detected on the folate deaminase glycoprotein. One type displays high affinity and binds folate stronger than N\textsuperscript{5}-methylfolate. This binding site appears to be identical with the catalytic site of folate deaminase. The other type of binding site shows lower affinity but prefers N\textsuperscript{5}-methylfolate relative to folate. A similar preference for N\textsuperscript{5}-methylfolate was observed in chemotaxis tests pointing to the possibility that the second type of binding site is involved in chemotactic perception of folate compounds. Folate perception and deamination could thus be performed by activities residing on the same polypeptide.

Cells of Dictyostelium discoideum respond chemotactically to cAMP (1) as well as to folate (2) and pterin (3). cAMP is the attractant during aggregation of differentiated cells; cells in the aggregation center produce cAMP signals (4–6) which stimulate neighboring cells to move toward the cAMP source (1) and to relay the cAMP signals outward in the aggregation field (7–10). In contrast to cAMP, folate and pterins may serve as attractants during food seeking of vegetative cells which naturally prey on folate- and pterin-releasing bacteria in the soil (3). The Dictyostelium chemosensory system includes enzymes which inactivate their cognate chemotactants, thereby reducing background concentrations and enabling cells to sense small concentration changes (11). cAMP is hydrolyzed to 5'-AMP by cAMP phosphodiesterase (12, 13), and folate and pterin are hydrolytically deaminated to the corresponding lumazine forms by means of folate and pterin deaminases (12, 14). Both cAMP phosphodiesterase and folate deaminase occur as extracellular and membrane-bound forms (12–14).

D. discoideum cells respond with different sensitivity to different folate compounds; as low as 10\textsuperscript{-6} M N\textsuperscript{5}-methylfolate can elicit a cellular response, whereas 10\textsuperscript{-4} M folate is required to stimulate the cells. 2-Deamino-2-hydroxyfolic acid and amethopterin have virtually no chemoactive potency (15, 16). Perception of a chemoattractant involves binding to a specific cell-surface receptor and transduction of the extracellular situation to intracellular targets. Binding sites that may function as chemoreceptor for folate have been described to occur on the surface of D. discoideum cells (17–21). However, there is some controversy with respect to specificity and number of different types of folate binding sites (17–21). Also it is not clear to what extent folate binding to the catalytic site of folate-inactivating enzymes contributes to the binding data.

Whereas the cAMP receptor/phosphodiesterase system has recently been analysed in detail and the molecular structures of its components have now been determined (13, 22, 23), basic biochemical data on the membrane-bound folate receptor/deaminase machinery remain to be elucidated. We report here the purification of folate deaminase and folate binding sites from membranes of D. discoideum.

MATERIALS AND METHODS

Chemicals—Folic acid (pteroylmonoglutamic acid), digitation, bovine serum albumin, and Norit A were obtained from Serva, Heidelberg, Federal Republic of Germany (FRG). Amethopterin (methotrexate, 4-amino-4-deoxy-N\textsuperscript{5}-methylfolate), pterin (2-amino-4-hydroxypteridine), Nonidet P-40, leupeptin, chymostatin, p-chloromercuriphenylsulfonate (pCMS), methyl a-mannopyranoside, and concanavalin A-Sepharose were from Sigma, Munich, FRG. [3',5',7,9-\textsuperscript{3H}]Folic acid (specific activity, 7–16 X 10\textsuperscript{6} Bq/mmol) and [3',5',7,9-\textsuperscript{3H}]methotrexate (specific activity, 5.7 X 10\textsuperscript{6} Bq/mmol) were from Amersham Buchler, Braunschweig, FRG. Aminohexyl-Sepharose 4B and a Mono Q column were from Deutsche Pharmacia, Freiburg, FRG. Zwittergent 3-14 is a product of Calbiochem, Munich, FRG. Genapol was from Fluka, Buchs, Switzerland. N\textsuperscript{5}-Methylfolinic acid and 2-deamino-2-hydroxyfolic acid were generously supplied by Dr. R. B. Angier, American Cyanamide Co. (Lederle Laboratories Div., Pearl River, NY).

The purity of the folate acid-related compounds was analysed by paper chromatography on Whatman 1 Chr with 0.5% Na\textsubscript{2}CO\textsubscript{3} as solvent. Amethopterin (R<sub>f</sub>, 0.68 ± 0.05) was found to be contaminated with N\textsuperscript{5}-methylfolate (R<sub>f</sub>, 0.87 ± 0.03). Purification was achieved by paper chromatography. The spot containing amethopterin was cut out and eluted with water. Concentration was determined by ultraviolet absorption spectrometry using published extinction coefficients (24, 25). The same procedure was used to purify [\textsuperscript{3H}]N\textsuperscript{5}-methylfolate (25) which was prepared from [\textsuperscript{3H}]methotrexate by anaerobic alkaline treatment (24).

Folate-Sepharose was prepared by carbodiimide coupling of folate to aminohexyl-Sepharose 4B as described by Salter et al. (26).

Culture Conditions—Cells of D. discoideum strain Ax2 were grown at 23°C axenically in nutrient medium supplemented with 1.8% maltose (27). Cells were harvested at densities of 5–9 X 10\textsuperscript{9} ml\textsuperscript{-1} and washed twice with cold (4–8°C) 17 mM Sörensens phosphate buffer, pH 6.0 (ISP buffer).

Preparation of Membrane Fraction—Unless stated otherwise, preparation of membrane fraction and subsequent steps of solubilization and purification were performed at 0–4°C. Washed cells were resuspended at a density of 2 X 10\textsuperscript{9} ml\textsuperscript{-1} in SP buffer containing 0.1 mg/ml BSA, bovine serum albumin.
leupeptin and 0.1 mg/ml chymostatin. Under continuous stirring cells were lysed by adding an equal volume of an aqueous solution of digitonin (0.1 mg/ml). Stirring was continued for 15 min. Subsequently the particulate fraction was collected by centrifugation (15 min at 10,000 g). The supernatant was washed twice with ice-cold Tris-HCl, and finally resuspended at one-tenth the volume of the lysate in 20 mM Tris-HCl, pH 7.0, containing 2 mM EDTA and 2 mM diithiothreitol. In other experiments a membrane fraction was prepared by mechanical disruption of cells according to the procedure described by Das and Henderson (28).

Solubilization of Folate Binding Sites and Folate Deaminase—The stirred membrane fraction was adjusted to 1% (w/v) Nonidet P-40 by dropwise addition of an 11% (w/v) aqueous solution. In later experiments Nonidet P-40 was replaced by Zwittergent 3-14 which was added as a 16% (w/v) aqueous solution to yield a final concentration of 1%. Stirring was continued for 10 min. Subsequently the suspension was centrifuged for 40 min at 48,000 × g. The pellet was discarded and the supernatant (“membrane extract”) was centrifuged for an additional 2 h at 100,000 × g.

Chromatography on Concanavalin A-Sepharose and Folate-Sepharose—The supernatant of the ultracentrifugation step was applied to a concanavalin A-Sepharose column (2.4 × 7 cm) equilibrated with 20 mM Tris-HCl, pH 7.0, containing 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂. The column was washed with 100 ml of 50 mM sodium acetate, pH 5.0, containing 1% Genapol, 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂. Subsequently, the column buffer was changed to 20 mM Tris-HCl, pH 8.0, containing 1% Genapol, 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂, and glycrophores were eluted by means of 200 ml of a linear gradient of 0–1% methyl α-D-mannopyranoside in this buffer. Fractions of 8.5 ml were collected, and the flow rate was 15 ml/h. Peak fractions of folate deaminase and binding activity were pooled, diluted 4-fold with H₂O, and dialyzed twice for 1 h against 2 liters of 20 mM Tris-HCl, pH 7.1. This treatment was required in order to adsorb folate deaminase and binding activity to a folate-Sepharose column (2.4 × 1 cm) equilibrated with 20 mM Tris-HCl, pH 7.1, containing 0.1% Genapol. The column was washed with 50 ml of this buffer, and subsequently proteins were eluted with 140 ml of a linear gradient of 0–200 mM KC1 in this buffer. Fractions of 4 ml were collected, and the flow rate was 6 ml/h.

Chromatography on Fast Protein Liquid Chromatography Mono Q—Active fractions from folate-Sepharose were dialyzed against 20 mM Tris-HCl, pH 8.5, 0.1% Genapol and applied to Mono Q. After washing with 10 ml of the dialysis buffer proteins were eluted with 40 ml of a linear gradient of 0–200 mM NaCl in this buffer. The purified protein eluted at about 100 mM NaCl.

Electrophoresis—Polyacrylamide gel electrophoresis in the presence of 15% SDS was performed according to Laemmli (29). Proteins were stained with Coomassie Brilliant Blue.

Protein Concentration Assay—The method of Hartree (30) was used with bovine serum albumin as standard. Where necessary protein was precipitated with acetone.

Purification of Folate Deaminase Activity—In the extract and in the column fractions folate deaminase activity was assayed with 0.1 mM folate by UV spectrophotometry at 355 nm (14). The temperature was 37 °C. In some experiments deamination of 10 nM [3H]folate was investigated at 0 °C. The technique involves separation of [3H]folate from [3H]-2-deamin-2-hydroxyfolate by paper chromatography and determination of their quantities by liquid scintillation counting (14). The same technique was used to assay folate deaminase activity with 100 nM [3H]folate and variable concentrations of unlabeled folate or its derivatives. These experiments were performed at 23 °C.

Determination of Folate Binding—Binding of folate to solubilized folate-binding proteins was determined by an assay based on the adsorption of free folate to Norit A coated with bovine serum albumin (Norit/BSA). The adsorption velocity of folate to Norit/BSA depended on the Norit concentration. At 0 °C a mixture of 150 mg/ml Norit A and 30 mg/ml BSA (final concentrations) adsorbed folate (20 nM and 200 μM) optimally (more than 98%) within 30 s. Detergents Nonidet P-40 (1%) and Zwittergent 3-14 (1%) did not disturb folate adsorption.

Folate deaminase activity present in the fractions was inactivated by pCMS (15 mM) during a preincubation period of 30 min at room temperature. This treatment was adjusted to 4 °C in a total volume of 300 ul containing 10 nM [3H]folate, 50 mM imidazole HCl, 1.6% Nonidet P-40 or 60 or 120 μl of pCMS-treated extract or column fraction, respectively. The mixture was incubated for 30 s to reach the binding equilibrium. Thereafter, 300 μl of ice-cold Norit/BSA (300 mg/ml Norit A and 60 mg/ml BSA) were added. After 30 s the suspension was centrifuged for 2 min in an Eppendorf centrifuge kept in a freezer at −5 °C. The supernatant, containing [3H]folate bound to binding proteins, was subjected to liquid scintillation counting. In other experiments the concentration of [3H]folate was varied, and variable concentrations of unlabeled folate or folate-related compounds were added. Radioactivity associated nonspecifically with the supernatant was determined in the presence of 0.2 mM unlabeled folate. The same technique was used to quantify binding of [3H]N7-methylfolate to solubilized binding proteins.

Dissociation of [3H]folate from folate binding sites was investigated by the addition of 0.2 mM unlabeled folate to the equilibrium mixture established from 10 nM [3H]folate. Dissociation proceeded approximately as a first order reaction with a rate constant of 6 × 10⁻5 s⁻¹ at 0 °C. Correspondingly, less than 5% of [3H]folate dissociated from the binding sites within 30 s, the incubation period with Norit/BSA.

RESULTS

Solubilization of Folate-binding Sites and Folate Deaminase—Crude membrane fractions were prepared either by chemical lysis of cells with a low concentration (0.05%) of digitonin or by mechanical disruption. From both membrane fractions similar quantities of folate binding sites and folate deaminase could be solubilized with specific detergents. Early experiments were performed with Nonidet P-40. Later we used Zwittergent 3-14 which solubilized slightly more folate binding sites and folate deaminase activity than Nonidet P-40.

Inactivation of Folate Deaminase—Quantification of folate binding in membrane extracts is affected by membrane-bound folate deaminase which degrades folate. Under the conditions of the standard binding assay (0 °C, 10⁻⁵ M [3H]folate) folate was almost completely deaminated to 2-deamino-2-hydroxyfolate during the incubation period of 30 s, and little binding of folate (or 2-deamino-2-hydroxyfolate) to protein could be measured. de Wit (19) used 8-azaguanine (51) to inhibit folate deaminase activity in suspensions of D. discoideum cells. Folate deaminase extracted from D. discoideum membranes, however, was not completely inhibited by 0.33 mM 8-azaguanine, the highest concentration applicable for reason of solubility. Here we describe inactivation of folate deaminase by p-chloromercuriphenylsulfonate. With increasing concentrations of pCMS, folate deamination was reduced to zero, and concomitantly the amount of folate molecules bound increased more than 10-fold (Fig. 1). In most binding experiments 15 mM pCMS were employed.

Purification of Folate-binding Sites and Folate Deaminase—The supernatant, obtained upon ultracentrifugation of the membrane extract, was chromatographed on concanavalin A-Sepharose (Fig. 2). Folate binding activity and folate deaminase activity both adsorbed to the lectin matrix. Neither activity was desorbed by high salt concentration (1 M NaCl). Elution was achieved with a gradient of methyl α-D-manno-
Flow through A B C

FIG. 2. Concanavalin A-Sepharose chromatography of folate deaminase and folate-binding sites. Elution was performed with a sequence of three steps: A, 50 mM sodium acetate, pH 5.0, containing 1% Genapol, 1 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂; B, 20 mM Tris-HCl, pH 8.0, containing 1% Genapol, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂; C, linear gradient of 0–1% methyl α-D-mannopyranoside in the buffer of B.

pyranoside. Surprisingly, folate binding sites and folate deaminase coeluted at the same mannoside concentration (Fig. 2). Peak fractions were pooled and adsorbed to folate-Sepharose (Fig. 3). Both activities were eluted by increasing KCl concentration. Again binding sites and deaminase activity were not separated. Binding activity was also assayed with 300 nM [³H]N⁰-methylfolate. These binding data (not shown) peaked in the same fractions as those obtained at 10 nM [³H]folate.

In other experiments peak fractions from the folate-Sepharose column were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Staining with Coomassie Blue revealed a single component with an apparent molecular mass of 138 kDa (Fig. 4). The intensity of Coomassie staining correlated with the activity of folate deaminase and folate binding in the corresponding fractions. These results suggest that folate deaminase and folate binding sites reside on the 138-kDa component. According to its lectin binding capacity, this component is a glycoprotein.

Although purification of the 138-kDa protein was possible solely by the use of concanavalin A and folate-Sepharose, concentration on fast protein liquid chromatography Mono Q was very useful and provided for additional purification in some cases (not shown). Table I summarizes the purification of folate deaminase and folate binding sites. Both activities were purified over 6,000-fold with respect to the crude membrane extract and (based on the activity of folate deaminase on cells (14)) in total, 25,000-fold. Following chromatography on folate-Sepharose and Mono Q the binding activity (measured in the presence of pCMS) was not as stable as the enzyme activity, resulting in a lower degree of purification.

Affinity and Number of Folate-binding Sites—The purified glycoprotein displayed a high affinity for folate whereas with N⁰-methylfolate as ligand a low affinity binding site was
Folate Recognition Protein from Dictyostelium Membranes

TABLE 1
Purification of folate-binding protein and folate deaminase

| Volume | Protein | Folate binding | Folate deamination |
|--------|---------|----------------|-------------------|
|        | ml      | mg             | Specific binding  | Purification | Yield | Specific activity | Purification | Yield |
| Membrane extract | 83 | 913 | 1.0 | 1 | 100 | 0.038 | 1 | 100 |
| Ultracentrifugation | 80 | 720 | 1.2 | 1.2 | 95 | 0.044 | 1.2 | 92 |
| Concanavalin A-Sepharose | 60 | 30 | 21 | 21 | 69 | 0.80 | 21 | 69 |
| Folate-Sepharose and Mono Q | 3 | 0.046 | 6025 | 6025 | 30 | 283 | 7447 | 37 |

Fig. 5. Dose-response curve of [3H] folate (●) and [3H]N⁰°-methylfolate (■) binding to the purified protein.

Fig. 6. Scatchard plot of [3H]folate (inset) and [3H]N⁰°-methylfolate binding to the purified protein. Data of three preparations are shown with different symbols. Number of molecules bound was calculated per 138-kDa protein; the ordinate is expressed as molecules bound/free (b/f, nM).

detected in addition to the high affinity binding site (Fig. 5). The apparent binding constants amounted to 4 nM for folate, and 8 and 170 nM for N⁰°-methylfolate, respectively. The affinity for folate of the low affinity binding site is at least 1 order of magnitude lower than the affinity to N⁰°-methylfolic. Accurate determination of the affinity to folate was not possible due to limitations of the binding assay. With increasing folate concentration the ratio of specific folate binding to nonspecific (background) binding decreases from up to 40 at 10⁻⁹ M to 0.3 and lower at 10⁻⁶ M. At ratios smaller than 0.3 binding data become increasingly inaccurate.

Determination of the number of binding sites per 138-kDa molecule of the high affinity site was not feasible due to the instability of the binding activity. With increasing folate concentration the ratio of specific folate binding to nonspecific (background) binding decreases from up to 40 at 10⁻⁹ M to 0.3 and lower at 10⁻⁶ M. At ratios smaller than 0.3 binding data become increasingly inaccurate.

Specificity of Folate-binding Sites and Folate Deaminase—

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Fig. 7. Competition of folate compounds for binding of 5 nM [³H]folate (A) or 300 nM [³H]N⁰°-methylfolate (B). Experiments were performed with the purified protein. ●, folate; □, N⁰°-methylfolate; Δ, 2-deamino-2-hydroxyfolate; ○, amethopterin. Levels of binding are presented as the percentages of the value obtained in the absence of any unlabeled compound.

We examined various unlabeled compounds for their capacity to inhibit binding of [³H]folate to the purified protein. Binding to the high affinity binding site was determined at 5 nM [³H]folate (Fig. 7A). 2-Deamino-2-hydroxyfolate competes for folate binding sites with an about 15-fold lower affinity than folate. Amethopterin, at concentrations of 0.1 and 1 μM, does not compete for folate binding. N⁰°-Methylfolate inhibits the binding of [³H]folate with an affinity slightly lower than that of folate. Analogous experiments were performed with 10 nM [³H]N⁰°-methylfolate and unlabeled folate or N⁰°-methylfolate. Consistent with the above result folate competed with higher affinity than N⁰°-methylfolate (not shown).

The low affinity binding site preferably binds N⁰°-methylfolate (Fig. 5). Competition experiments were performed at 0.3 μM [³H]N⁰°-methylfolate at which concentration about twice as many [³H]N⁰°-methylfolate molecules are bound to the low affinity sites than to the high affinity sites (Fig. 5). Folate inhibits binding of [³H]N⁰°-methylfolate with an affinity lower than that of N⁰°-methylfolate (Fig. 7B). 2-Deamino-2-hydroxyfolate competes only weakly for N⁰°-methylfolate binding. Amethopterin, at concentrations up to 30 μM, does not compete.
Since both folate deaminase activity and folate binding sites appear to reside on the same protein, one of the folate binding sites found in the presence of pCMS likely represents the catalytic site. To obtain information on this matter we determined the extent of inhibition of [3H]folate deamination by folate derivatives in the absence of pCMS (Table II). The relative potency of different derivatives to compete for folate deamination is similar to their potency to inhibit folate binding to the high affinity binding site (Fig. 7). These results suggest that the high affinity binding site corresponds to the catalytic site of the enzyme.

### DISCUSSION

We have purified to apparent homogeneity a 138-kDa glycoprotein from *D. discoideum* membranes which has folate deaminase activity and presumably contains the surface-bound folate deaminase of *D. discoideum* cells (14, 32). Deaminase activity could be effectively inactivated by the organomercurial pCMS, and this inactivation made possible the determination of specific folate binding to protein. A high affinity binding site, displaying higher affinity to folate than to N^10^-methylfolate, was detected in the deaminase fractions. The specificity of this binding site is similar to the specificity of deaminase activity. Differences in the specificities may be due to different assay conditions, 5 nM [3H]folate and 15 mM pCMS in the binding assay as compared with 100 nM [3H]folate and no pCMS in the deaminase activity assay. We conclude that the high affinity binding site is the catalytic site of folate deaminase. It follows that pCMS inhibits catalysis but not binding to the catalytic site.

In addition to the high affinity binding site two low affinity binding sites have been found on the 138-kDa glycoprotein. These low affinity binding sites preferentially bind N^10^-methylfolate. Both, the high affinity and the low affinity binding site were specific for folate compounds and showed no measurable affinity for pterin.

The major folate binding site detectable on cells displays high affinity for amethopterin (17, 20). This amethopterin binding component differs from the folate binding component described here, which, according to the competition data of Fig. 7, does not bind amethopterin. Already in the crude extract amethopterin binding was absent. Therefore, the amethopterin binding component either could not be solubilized by Zwittergent 3-14 and Nonidet P-40 or became inactive upon solubilization. The function of the amethopterin binding component is not clear. Since amethopterin is not a chemoattractant (15, 16), the amethopterin binding component apparently is not involved in chemotactic perception of folate compounds.

The low affinity binding sites could be additional catalytic or regulatory sites of the deaminase required for the appropriate control of signal destruction. An alternative view is the following. In chemotaxis tests and in a light scattering assay (which measures responses related to chemotaxis) *D. discoideum* cells respond more sensitively to N^10^-methylfolate than to folate (15, 16). Comparison of these results with our binding data points to the possibility that the low affinity binding site is involved in chemotactic perception of folate compounds. It is true the affinity of this binding site is much lower than the chemotactic affinity to N^10^-methylfolate (16) and, at the first glance, this argues against the proposed relation. However, the affinity of a receptor may change upon solubilization and separation from interacting membrane proteins. For instance, the affinity of the β-adrenergic receptor is reduced after purification and increases dramatically upon interaction with a signal-transducing GTP-binding protein (33). In agreement we found that in the crude extract folate binding was increased in the presence of GTP (2) de Wit and van Haastert (20) assigned the chemotactic specificity to B sites which, in contrast to the A sites, exhibit a slow dissociation of folate compounds of $2 \times 10^{-3}$ s^{-1}. Using their assay conditions we found a similar dissociation constant of $2.5 \times 10^{-3}$ s^{-1} for the purified protein, suggesting that the deaminase is part of the chemotaxis receptor protein. However, further studies are required to show whether reconstitution of the protein into membrane vesicles yields the expected affinity for folate and whether disruption of the gene for the 138-kDa protein will indeed abolish both deaminase activity and chemotaxis.

Our conception implies that *D. discoideum* has developed different molecular strategies to deal with different chemostimuli; extracellular cAMP signals are sensed and cleared by distinct molecules, a 40-kDa membrane receptor (22), and a membrane-associated phosphodiesterase which also occurs in a soluble, extracellular form (13, 23). In contrast, folate perception and background reduction may both be performed by a bifunctional receptor deaminase protein. The difference at the receptor level may be connected with the involvement of different GTP-binding proteins in the transduction of folate and cAMP signals, respectively (34). Beyond the GTP-binding proteins the signal transduction pathways appear to converge, since stimulation with folate as well as with cAMP elicits common reactions in the cells, for example, a rapid transient increase in the intracellular cGMP concentration (35–37).

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