# Two distinct types of cell surface folic acid-binding proteins in *Dictyostelium discoideum*

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Folic acid is degraded too fast by *Dictyostelium discoideum* to study binding of this ligand to cell surface binding proteins. Folate deaminase activity was inhibited in the presence of $3.3 \times 10^{-4}$ M 8-azaguanine. This inhibitor enabled us to detect two folate binding proteins. One type bound folic acid and deamino-folic acid with the same affinity ($K_{0.5} = 3\text{–}6 \times 10^{-7}$ M) and apparently negative cooperativity. Binding to only this type was observed if 8-azaguanine was omitted. The second type bound folic acid noncooperatively with $K_d = 7 \times 10^{-7}$ M. Deamino-folic acid did not compete even at a 1000-fold excess. This type may correspond to the chemotactic receptor.

*Folic acid* *Deamino-folic acid* *Binding proteins* D.discoideum

## 1. INTRODUCTION

Cellular slime mold species respond chemotactically to folic acid (FA) [1] and in general are more sensitive to this attractant in the vegetative stage than in the aggregative stage. Pulses of FA induce synthesis and secretion of folate deaminase and phosphodiesterase in cell suspensions of *Dictyostelium discoideum* [2]. Both enzymes are involved in the degradation of the chemoattractants FA and cAMP. FA is degraded rapidly by several extracellular and membrane-bound deaminases [3–5]. The extracellular molecular signals for chemotaxis and induction of enzymes are thought to be detected by cell-surface binding proteins or receptors. Much effort has been spent on the characterization of folate binding in *D.discoideum* [6–8]. One type of binding protein has been reported, which binds both FA and 2-deamino-FA (DAFA) and yields nonlinear Scatchard-plots, either indicating heterogeneity or negative cooperativity. A $K_{0.5}$ of $1.5\text{–}3 \times 10^{-7}$ M has been reported [6,7].

Since DAFA is at least 10000-fold less active than FA as a chemoattractant and DAFA is not an antagonist for chemotaxis towards FA [9], DAFA is unlikely to bind to the chemotactic receptor. Thus the receptor, which is known to bind both FA and DAFA, may mediate processes other than chemotaxis.

FA binding studies have been done without preventing deamination or using labelled methotrexate [8], which is not degraded by the deaminase(s) of *D.discoideum*. Here, I report the detection of a second binding protein, which is specific for FA, using the deaminase inhibitor 8-azaguanine [12–14].

## 2. EXPERIMENTAL

[7,9,3′,5′-$^3$H]Folic acid was purchased from Amersham Radiochemical Centre (Bucks). [7,9,3′,5′-$^3$H]DAFA was prepared by a 2 h incubation of tritiated FA in the dark at 20°C with extracellular deaminase from *D.lacteum*, which was obtained as in [10]. The product contained <0.1% folic acid as judged by reverse-phase HPLC. Folic acid was purchased from BDH biochemicals, pterin-6-carboxylic acid and polyethylene glycol 6000 from Sigma Chemical Co. and 8-azaguanine from Fluka AG (Buchs). DAFA was prepared enzymatically and purified as in [9].

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2.1. *Culture conditions*

*Dictyostelium discoideum* NC4 (H) was cultivated on SM-agar [11] together with *Escherichia coli* B/r. In the late logarithmic phase cells were harvested and washed 3 times by centrifugation at $150 \times g$ during 4 min in cold 10 mM phosphate buffer (pH 6.5) and adjusted to $5 \times 10^7$ cells/ml and the suspension was aerated at 0°C during 5 min before use in binding experiments.

2.2. *Binding assay*

Cells were prepared as above. Final concentrations in the incubation mixture were: $3.3 \times 10^7$ cells/ml, $6.7 \times 10^{-8}$ M [$^3$H]FA or [$^3$H]DAFA (both 6000 cpm/pmol), $3.3 \times 10^{-4}$ M 8-azaguanine and various [FA] or [DAFA] from $10^{-8}$–$10^{-4}$ M in 10 mM phosphate buffer (pH 6.5). Binding equilibrium was reached within 1 min at 0°C, during which time the samples were layered on top of 150 $\mu$l 12% polyethylene glycol (PEG) in 1.6 ml centrifugation tubes. Incubation was terminated after 1 min by centrifugation at $8000 \times g$ during 30 s; the supernatant was aspirated and the tubes were wiped dry. The pellet was resuspended in 100 $\mu$l $H_2O$, mixed with 3 ml Instagel and radioactivity was measured.

2.3. *Deaminase assay*

Samples were prepared as above. Incubation was done at various [8-azaguanine] during 1 min, after which 500 $\mu$l of an ice-cold suspension of SP-Sephadex in 10 mM $NH_4$/HCOO (pH 2.0) was added. (The volume of the settled resin was 50% of the total volume.) After centrifugation for 1 min at $8000 \times g$, radioactivity was determined in 300 $\mu$l supernatant containing the product ([$^3$H]DAFA).

## 3. RESULTS

Without 8-azaguanine, $6.7 \times 10^{-8}$ M FA was degraded ~90% under binding assay conditions. This observation implies that binding of FA by cells will be underestimated due to the action of membrane-bound deaminase. Instead, binding of DAFA will be observed.

In the presence of $3.3 \times 10^{-4}$ M 8-azaguanine, FA degradation was reduced to <5% during 1 min incubation (fig. 1). The inhibition curve was very shallow and complex, which may be caused by the heterogeneity of the deaminase activity.

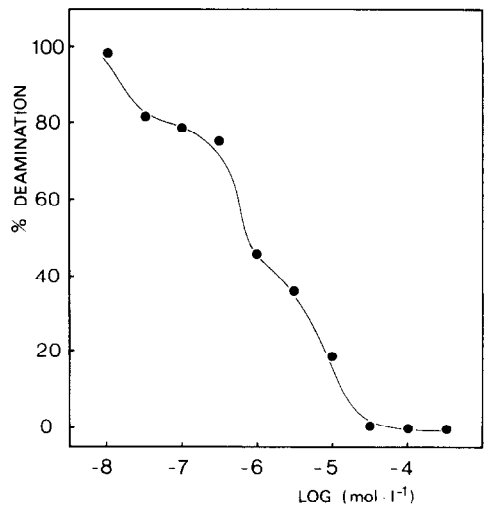


Fig. 1. FA degradation under binding assay condition at various [8-azaguanine].

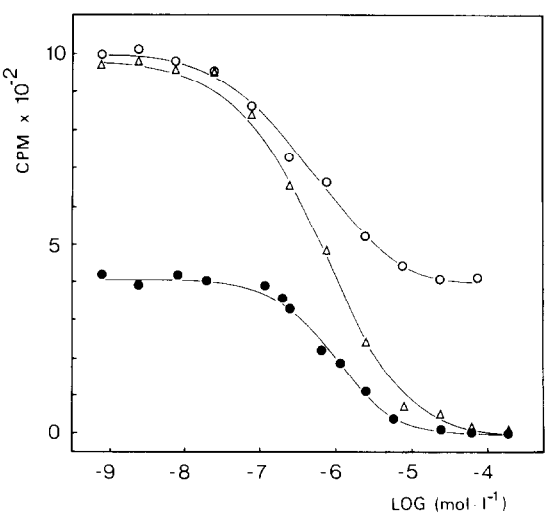


Fig. 2. [$^3$H]FA radioactivity retained by vegetative cells; (—○—) competition with DAFA; (—△—) competition with FA; (—●—) competition with FA in the presence of $2 \times 10^{-5}$ M DAFA. All other conditions were described in section 2.2.

Binding studies revealed two types of folate binding proteins. For one type DAFA competed with bound [$^3$H]FA (fig. 2); $>2 \times 10^{-5}$ M DAFA did not reduce binding of [$^3$H]FA further, although still 40% of the original amount of bound FA was still present. This amount could only be

Table 1

| Radioligand ($6.7\times10^{-8}$ M) | DAFA ($2\times10^{-5}$ M) | 8-Azaguanine ($3.3\times10^{-4}$ M) | Bound[a] (cmp) |
|---|---|---|---|
| [$^3$H]FA | 0 | + | 1060 ± 65 |
| [$^3$H]FA | + | + | 450 ± 30 |
| [$^3$H]FA | 0 | 0 | 610 ± 30 |
| [$^3$H]DAFA | 0 | + | 590 ± 30 |
| [$^3$H]DAFA | + | 0 | 5 ± 10 |

[a] The blank value or aspecific binding was usually 100 ± 5 cpm, as determined in the presence of $2 \times 10^{-4}$ M FA

reduced through competition with unlabelled FA.

Table 1 gives the amount of radioactivity bound to both binding proteins in the presence and absence of 8-azaguanine and using [$^3$H]DAFA instead of [$^3$H]FA. It is obvious that either omitting 8-azaguanine or using [$^3$H]DAFA yielded binding to only the aspecific binding protein; i.e., the type binding both FA and DAFA.

The aspecific binding protein bound DAFA and FA with equal affinity (fig. 3) and apparently negative cooperativity ($n_H = 0.90$, fig. 4). The $K_{0.5}$ varied from $3–6 \times 10^{-7}$ M using different batches of cells. About 200000 binding sites/cell were detected in vegetative cells.

The FA-specific binding protein bound FA with $K_d$ $7 \times 10^{-7}$ M. A 1000-fold excess of DAFA did not compete. No cooperativity was observed and ~100000 binding sites/cell were present in the vegetative stage. Addition of $2 \times 10^{-4}$ M pterin-6-carboxylic acid did not reduce [$^3$H]FA binding to both types of binding proteins, indicating that these proteins are specific for folates and do not bind pterin derivatives.

Furthermore pterin-6-carboxylic acid is a good substrate for the deaminase [5] of *D.discoideum* and should thus prevent binding of [$^3$H]FA to the membrane-bound deaminase(s), except to the type that is specific for FA [4].

## 4. DISCUSSION

Until now FA binding in *D.discoideum* has been studied without preventing deamination. As shown here, the inhibition of deaminase activity resulted in detection of a second type of FA binding protein. This type bound FA with high affinity and non-cooperatively, while DAFA was not bound even at 1000-fold higher concentrations.

The binding protein which has been studied before bound FA and DAFA with the same high affinity and negative cooperativity or heterogeneity [6,7]. This corresponds well to the results presented

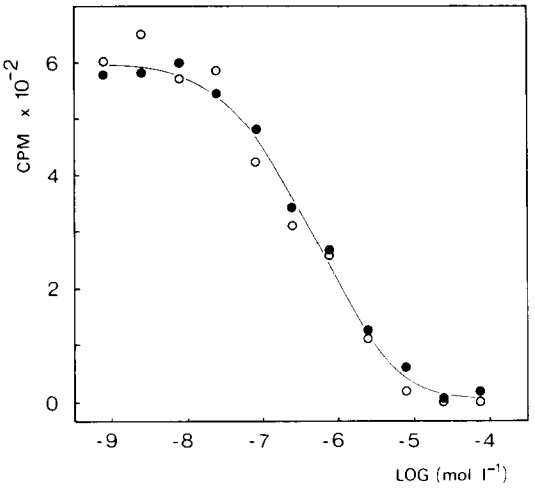


Fig. 3. [$^3$H]DAFA radioactivity retained by vegetative cells; (—○—) competition with DAFA; (—●—) competition with FA.

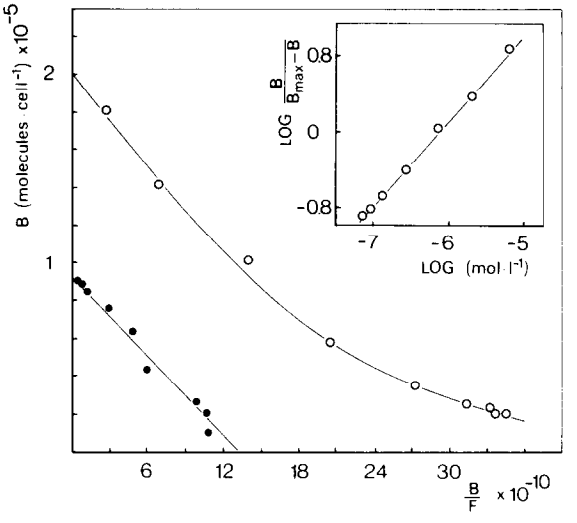


Fig. 4. Scatchard plot of [$^3$H]FA binding (—●—) obtained in the presence of $2 \times 10^{-5}$ M DAFA, and of [$^3$H]DAFA binding (—○—). Inset: Hill plot of DAFA binding; $B$ in molecules/cell; $F$ in mol/l.

here for the FA/DAFA aspecific protein. The discrepancy in the no. binding sites/cell ($4 \times 10^4$ [8]; $6 \times 10^4$ [6]) may have been caused by dissociation during the separation of bound from unbound ligand; e.g., a larger volume of silicon oil or PEG would cause longer sedimentation times and thus more dissociation. This effect was also observed in these experiments.

Methotrexate (MTX) which is not a substrate for the deaminase(s) of *D.discoideum,* was used as ligand in [8]. Only one binding protein was observed. FA was ~50-times less effective as a competitor than MTX, which implies that, if the $K_d$ for MTX is $\sim 5 \times 10^{-8}$ M, the $K_d$ for FA is $\sim 3 \times 10^{-6}$ M. However, under the binding conditions used, FA should have been deaminated completely. Thus competition by DAFA was studied instead. Since DAFA bound relatively well to this protein, when compared to the FA specific protein here ($K_d \geqslant 7 \times 10^{-6}$ M) it is more likely that MTX binding to the aspecific binding protein was studied.

Since 8-azaguanine inhibited folate deaminase activity, it might also interact with folate binding proteins. From table 1 it is clear that no effect of 8-azaguanine on binding to the FA/DAFA-aspecific binding protein was seen. An effect on the FA-specific protein can not be excluded.

It is known that DAFA possesses no chemotactic activity in *D.discoideum.* Since DAFA binds very well to one type of membrane-bound binding protein, this protein may not be involved in chemotaxis or DAFA should have antagonistic properties. The second alternative was excluded in [9]. In contrast, the binding protein, which is specific for FA, may well be the chemoreceptor for FA; i.e., the membrane-bound receptor protein mediating chemotaxis.

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