Quantitative description of the interaction between folate and the folate-binding protein from cow's milk

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A detailed study has been carried out on the dependence of folate binding on the concentration of FBP (folate-binding protein) at pH 5.0, conditions selected to prevent complications arising from the pre-existing self-association of the acceptor. In contrast with the mandatory requirement that reversible interaction of ligand with a single acceptor site should exhibit a unique, rectangular hyperbolic binding curve, results obtained by ultrafiltration for the FBP–folate system required description in terms of (i) a sigmoidal relationship between concentrations of bound and free folate and (ii) an inverse dependence of affinity on FBP concentration. These findings have been attributed to the difficulties in determining the

free ligand concentration in the FBP–folate mixtures for which reaction is essentially stoichiometric. This explanation also accounts for the similar published behaviour of the FBP–folate system at neutral pH, which had been attributed erroneously to acceptor self-association, a phenomenon incompatible with the experimental findings because of its prediction of a greater affinity for folate with increasing FBP concentration.

Key words: acceptor self-association, bovine milk, folate-binding protein, folate interaction, stoichiometric interaction, ultrafiltration binding study.

INTRODUCTION

Attempts to ascribe an affinity to the interaction between folate and the single site on FBP (folate-binding protein) from cow's milk have been inconclusive, since the previously reported dissociation constants at neutral pH have exhibited a dependence on the concentration of FBP used for their determination [1–3]. Furthermore, the reported dissociation constants merely refer to the folate concentration for half-maximal binding, since evaluation of an actual equilibrium constant was precluded by the sigmoidal nature of the binding curves: in some situations, the co-operativity was reported to be negative [2–5]. Under slightly acidic conditions (pH 5), the interaction is more closely described by a rectangular hyperbolic dependence of folate binding on free folate concentration [2,3]. On the grounds that FBP undergoes self-association at neutral pH [2,3,6], but not at pH 5 [3], the co-operativity of binding has been attributed [2,3] to an interplay between ligand binding and acceptor self-association that is either pre-existing [7] or ligand-mediated [8,9].

The initial emphasis of the present study was on the dependence of the folate-binding curve on FBP concentration at pH 5.0, conditions selected to prevent complications arising from self-association of the protein. Since the results of these ultrafiltration studies on folate binding also exhibit the symptoms of co-operativity and acceptor-concentration dependence found [2,3] at neutral pH, FBP self-association is precluded as the source of the unusual binding behaviour. A solution to the dilemma is seemingly provided by an alternative analysis of the present and published [3] results, which have established their determination from mixtures in which the folate–FBP interaction is essentially stoichiometric.

EXPERIMENTAL

Folic acid was obtained from Sigma (St. Louis, MO, U.S.A.) and its tritium-labelled derivative was from the Radiochemical Centre

(Amersham, U.K.). Folate–agarose affinity adsorbent was prepared from Affigel-102 (Bio-Rad) by covalent coupling of the ligand with the amino group of DEAE–agarose.

Purification of FBP

FBP was purified from spray-dried whey protein concentrate (Bonlac, Melbourne, Australia) by an adaptation of the affinity chromatography procedure using folate as the immobilized ligand [2,10]. This analogous procedure [11] produces FBP in a yield (38 %) comparable with the recoveries of FBP activity (25–41 %) obtained by earlier affinity chromatography methods [2,10,12].

The purity of the FBP preparation can be gauged from the elution profile obtained by exclusion chromatography on Sephacryl S-200 (Figure 1, lower trace). From the upper elution profile, which reflects the exclusion chromatography of a sample supplemented with 150 μ M folate and a tracer amount of [³H]folate, it is evident that the folate-binding capacity is restricted to the major protein zone. The fact that the faster-migrating species, constituting less than 10 % of the preparation, do not reflect aggregated forms of FBP was also indicated by their absence from the elution profiles obtained by rechromatography of material from the major fraction on Sephacryl S-200. The molecular mass of the purified FBP (~30 kDa) was confirmed by SDS gel electrophoresis.

In ultracentrifuge and exclusion chromatography studies, the concentrations of FBP solutions were based on an absorption coefficient ($A_{1\text{cm}}^{1\%}$) of 2.0 at 280 nm, which was determined in this laboratory. The discrepancies between our estimate and the reported absorption coefficients of 1.8 [2] and 2.35 [13] could well reflect differences in carbohydrate content of the respective preparations.

For ligand-binding studies, the concentrations of FBP solutions were determined spectrofluorimetrically by stoichiometric titration with *N* $^{\alpha}$ -pteroyl-*N* $^{\epsilon}$ -(4'-fluorescein-thiocarbamoyl)-L-lysine [14], a lysine analogue of folic acid. As in previous studies [2,3], the FBP concentrations were based on binding capacity.

Abbreviation used: FBP, folate-binding protein.

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Figure 1 Exclusion chromatography of FBP

Elution profiles for purified FBP (1 ml, ~1 mg/ml) on a Sephacryl S-200 column (1.5 cm × 68 cm) equilibrated with 0.2 M acetate buffer (pH 5.0) and operated at a flow rate of 10 ml/h. The upper profile refers to an experiment in which the applied sample was supplemented with 150 μ M folate (plus tracer amounts of tritium-labelled folate) to obtain additionally an elution profile for folate-bound FBP (●).

Sedimentation equilibrium studies

Before centrifugation, solutions of FBP were dialysed extensively against either 0.2 M phosphate buffer (pH 6.8) or 0.2 M acetate buffer (pH 5.0). The dialysed protein solutions were then spun at 17000 rev./min and 20 °C in a Beckman XL-I ultracentrifuge for 24 h, by which time sedimentation equilibrium had been attained. Equilibrium distributions reflecting low initial FBP concentrations were monitored spectrophotometrically at 280 nm, whereas those obtained for higher loading concentrations (0.5–5 mg/ml) were recorded interferometrically in terms of the number of Rayleigh fringes $j(r)$, which ranged from 0 at the air–liquid meniscus (located at radial distance r_a) to $j(r_b)$ at the cell base. Since the sedimentation equilibrium experiments were of low-speed design [15], the conversion of these $j(r)$ distributions into the Rayleigh counterpart of the absolute concentration distribution, $J(r)$ versus r , entailed the determination of $J(r_a)$, which is the number of Rayleigh fringes corresponding to the protein concentration at the air–liquid meniscus, by the M^* procedure [16,17]. The absolute fringe number at radial distance r was then calculated as $J(r) = J(r_a) + j(r)$, which allowed the delineation of the mass–concentration distribution, $c(r)$ versus r , using the relation $c(r) = J(r)/3.33$ [18]. The corresponding concentration distributions in the spectrophotometrically recorded experiments were based on the absorption coefficient of 2.0 for FBP (see above). A partial specific volume $\bar{v}$ of 0.713 ml/g [2] was used in the buoyancy correction factor $(1 - \bar{v}\rho)$, where ρ is the buffer density.

Ligand-binding studies

Centrifugal ultrafiltration [19] was used to quantify the binding of folate to FBP. Mixtures (0.4 ml) of FBP and [3 H]folate (0.3–1.0 Ci/mmol) in either 0.2 M acetate buffer (pH 5.0) or 0.2 M phosphate buffer (pH 7.3) were placed in the cups of Microcon-30

Table 1 Summary of molecular mass estimates for FBP from sedimentation equilibrium experiments at 20 °C and 17 000 rev./min

Numbers in parentheses denote the uncertainty (± 2 S.D.) of the estimates.

Optical system	Concentration range (mg/ml)	Molecular mass (kDa)
Phosphate buffer (0.2 M, pH 6.8)		
Absorption	0.01–0.36	33.0 (± 1.2)
Absorption	0.01–0.44	34.0 (± 1.0)
Absorption	0.03–1.22	36.0 (± 0.8)
Acetate buffer (0.2 M, pH 5.0)		
Absorption	0.02–0.18	28.2 (± 0.2)
Absorption	0.03–0.31	28.2 (± 0.2)
Absorption	0.03–0.31	27.8 (± 0.3)
Interference	0.07–1.10	27.8 (± 0.2)
Interference	0.34–4.83	27.1 (± 0.1)
Interference	0.91–7.50	26.8 (± 0.1)

ultrafiltration devices (Millipore) and incubated at 22 °C for 90 min before the collection of a sample of ultrafiltrate by centrifugation. Duplicate aliquots of each ultrafiltrate were then mixed with Ready-Protein scintillation fluid (Beckman) to allow assessment of their radioactivities in a Beckman LS 3801 scintillation counter calibrated by means of an external standard. These measurements provided an estimate of the free folate concentration $[\text{folate}]_{\text{free}}$ in a mixture with total ligand concentration $[\text{folate}]_{\text{total}}$. The concentration of bound ligand $[\text{folate}]_{\text{bound}}$ was then taken as the difference between $[\text{folate}]_{\text{total}}$ and $[\text{folate}]_{\text{free}}$. Binding curves, $[\text{folate}]_{\text{bound}}$ versus $[\text{folate}]_{\text{free}}$, were determined for a series of mixtures containing two fixed concentrations of FBP (0.77 and 66 μ M) under each set of conditions (0.2 M acetate, pH 5.0; 0.2 M phosphate, pH 7.3).

RESULTS AND DISCUSSION

As noted in the Introduction section, a pH value of 5.0 was selected for the initial part of the present investigation on the grounds that studies of folate binding to FBP under those conditions should be free from complications arising from acceptor self-association. The ligand-binding studies were therefore preceded by a check on the macromolecular state of FBP.

Macromolecular state of FBP

Although FBP eluted as a single symmetrical zone in exclusion chromatography on Sephacryl S-200 (Figure 1), the elution volume was commensurate with a molecular mass of 44 kDa, which is considerably larger than the accepted value (~30 kDa) for monomeric FBP that is obtained by exclusion chromatography at pH 6.8 (results not shown). Therefore sedimentation equilibrium studies (17000 rev./min, 20 °C) were performed to obtain more certain estimates of the molecular mass. As reported earlier [2,3,6], FBP undergoes self-association at neutral pH (entries 1–3 in Table 1). Despite the return of a single molecular mass because of the close conformity of each equilibrium distribution with a linear dependence of $\ln A_{280}(r)$ on the square of radial distance, self-association is indicated by the increase in molecular mass estimate M , with the mean of the concentration range covered by the sedimentation equilibrium distribution. The value of M , a mass-average parameter, is also significantly higher than the molecular mass of 27–28 kDa, which is deduced from the amino acid sequence [20] and the carbohydrate content [2] of bovine FBP.

Figure 2 Binding curves for folate-FBP interaction at pH 5.0

(A) Results (●) from ultrafiltration studies (22 °C) with 0.77 μM FBP, together with best-fit descriptions in terms of co-operative binding (—) and a rectangular hyperbolic dependence on free folate concentration (---). (B) Results (●) from studies with 66 μM FBP and their best-fit description (—) in terms of co-operative binding and folate partition.

The behaviour of FBP at pH 6.8 is markedly different from that deduced from sedimentation equilibrium distributions obtained for the protein at pH 5.0 (entries 4–9 in Table 1). Extrapolation of the reciprocals of these apparent molecular masses to zero FBP concentration yields the respective estimates of 28 000 (± 200) Da and $3.6 (\pm 1.2) \times 10^{-4} \text{ ml} \cdot \text{mol}^{-1} \cdot \text{g}^{-2}$ for the molecular mass and second virial coefficient under these conditions. Thus these results substantiate earlier findings [2,3,6] that FBP does not self-associate under these conditions and thereby identify the molecular mass deduced from the elution volume as an incorrect result.

Elution volumes in exclusion chromatography are traditionally interpreted in terms of anhydrous molecular mass [21,22], whereas the parameter being monitored is the solvated (hydrated) molecular volume [23]. Thus an overestimation of molecular mass by exclusion chromatography of FBP at pH 5.0 could, in principle, reflect a greater than usual extent of solvation under the slightly acidic conditions. In that case, the increase in volume for monomeric FBP between pH 6.8 and 5.0 should be approx. 1.3-fold (25% increase in Stokes radius), which is rather large but still less than the 2-fold increase (47% increase in Stokes radius) associated with the acid expansion of BSA [24–30].

Folate-binding studies at pH 5.0

Results of an ultrafiltration study on folate binding to 0.77 μM FBP in acetate buffer (pH 5.0) are summarized in Figure 2(A).

Analysis of bound folate in terms of a rectangular hyperbolic dependence on free ligand concentration yields an apparent dissociation constant K_d of $0.06 \pm 0.01 \mu\text{M}$, which is approx. 8-fold higher than the estimate obtained by equilibrium dialysis with 0.1 μM FBP [2]. Although the uncertainty ($\pm 2\text{S.D.}$) of the current estimate is seemingly satisfactory, the results are poorly described by that best-fit description (Figure 2A, broken line). In keeping with published studies at neutral pH [2,3], a much better description (solid line) is obtained by invoking co-operative binding of folate (a Hill coefficient n of 1.69 ± 0.17). An increase in the FBP concentration to 66 μM leads to data (Figure 2B) that require description in terms of folate binding and a second event that is either physical partitioning or extremely weak binding of folate (the essentially linear section of Figure 2B). For these results, there is no adequate description in terms of a simple binding phenomenon for the stronger event and, hence, the indicated dependence (solid line) reflects the outcome of curve-fitting in terms of co-operative binding ($n = 1.44 \pm 0.26$) and a partition factor (0.031 ± 0.004).

A problem with invoking the co-operativity concept to provide a better description of the experimental data is the scarcity of physically realistic models that can give rise to such behaviour for a non-associating acceptor with a single ligand-binding site. Although self-association of the FBP-folate complex has the potential to generate sigmoidal binding behaviour [8], this possible explanation is precluded on two grounds. First, the FBP-folate complex exhibited the same elution volume as folate-free FBP in exclusion chromatography on Sephacryl S-200 (Figure 1). Secondly, the predicted strengthening of ligand binding by an increase in acceptor concentration [8] is the reverse of that indicated by the corresponding dependence of free ligand concentration associated with half-maximal binding (0.05 μM for 0.77 μM FBP; 1.25 μM for 66 μM FBP). Therefore an interplay between folate binding and self-association of FBP cannot be invoked to account for the results of these folate-binding studies at pH 5.0.

In the absence of any satisfactory interpretation of the results (Figure 2) in terms of the ligand-binding theory, we seek an alternative explanation of their origin. To that end, the results are replotted in Figure 3 as a dependence of bound folate concentration on its total counterpart. This is the format used to present the results of stoichiometric titrations in which the reactant concentrations are sufficiently high to render complex and the excess free reactant as the sole species in solution. Several points are noted in relation to Figure 3. (i) The results of both experiments are well described by two linear segments. Indeed, application of the F -test signifies superiority of such descriptions of the data over those for the co-operative model (Figure 2). (ii) Since the concentration of bound folate has been deduced from the difference between total concentration and the concentration of the ultrafiltrate, the slopes of the initial linear segments in Figures 3(A) and 3(B) are necessarily less than the value of unity associated with stoichiometric titrations. However, the respective values of 0.90 and 0.93 in Figures 3(A) and 3(B) are sufficiently close to unity to warrant consideration of the possibility that there has been a slight overestimation of $[\text{folate}]_{\text{free}}$ by the ultrafiltration procedure. (iii) Although preliminary tests were performed to check that FBP was retained by the ultrafiltration membranes (30 kDa cut-off), a small extent of protein leakage through the membrane would account for the measured folate concentrations in the ultrafiltrate under conditions commensurate with stoichiometric folate-FBP interaction.

In view of the proximity of the molecular mass of FBP to that (30 kDa) designated as the limit for impermeability of the membrane, a limited extent of acceptor contamination of the

Figure 3 Alternative analysis of folate–FBP binding data (pH 5.0)

Test of the binding data from Figure 2 for conformity with the concept of stoichiometric complex formation between folate and FBP. Results were obtained with (A) 0.77 μM and (B) 66 μM FBP.

ultrafiltrate is possible. We therefore consider that our folate-binding studies at pH 5.0 cannot be interpreted in terms of an equilibrium constant because of essentially stoichiometric interaction of the ligand with FBP under these conditions.

Folate-binding studies at neutral pH

Results of ultrafiltration studies on folate binding to 0.77 and 66 μM FBP in phosphate buffer (pH 7.3) are summarized in Figure 4. From the dependence of $[\text{folate}]_{\text{bound}}$ on free folate concentration, it is evident that these results also required description in terms of co-operative binding ($n = 1.64$ and 1.53 in Figures 4A and 4B respectively), without the necessity to invoke a second (partition) phenomenon. As also observed in our studies of the interaction at pH 5.0 (Figure 2), the free folate concentration associated with half-maximal binding increases with the concentration of FBP [0.04 μM for mixtures containing 0.82 μM FBP (Figure 4A) and 2.7 μM for those with 55 μM protein (Figure 4B)]. In both respects, the current results emulate the findings obtained from equilibrium dialysis studies of the folate–FBP interaction under similar conditions [2,3].

The occurrence of FBP self-association in the absence of folate at neutral pH (Table 1, see also [2,3,6]) raises the possibility that the sigmoidal (co-operative) binding behaviour could reflect an interplay between ligand binding and acceptor self-association that is either pre-existent [7] or ligand-mediated [8]. However, since the above observation of weakened interaction after increase in FBP concentration is incompatible with either model, we again examine the results in the context of a possible stoichiometric

Figure 4 Binding curves for folate–FBP interaction at pH 7.3

Best-fit descriptions (—) of results (●) from ultrafiltration studies of the folate–FBP interaction at neutral pH (0.2 M phosphate buffer, pH 7.3) in terms of co-operative binding. (A) $[\text{FBP}]_{\text{total}} = 0.82 \mu\text{M}$, $n = 1.64$; (B) $[\text{FBP}]_{\text{total}} = 55 \mu\text{M}$, $n = 1.53$. (C, D) The corresponding analyses in terms of stoichiometric interaction.

Figure 5 Stoichiometric analyses of published data for the folate-FBP interaction

Dependence of bound folate on the total folate concentration in equilibrium dialysis experiments, the results (●) being inferred from Figures 3–5 of [3]. (A) $[FBP]_{total} = 0.23$ nM; (B) $[FBP]_{total} = 8.2$ nM; (C) $[FBP]_{total} = 2.2$ μ M. Solid lines denote the theoretical dependencies calculated for a system with $K_d = 0.02$ nM, which is the dissociation constant obtained from the experiment with the lowest FBP concentration (see Table 1 of [3]); the broken line in (A) reflects the consideration of that experiment also in terms of stoichiometric interaction.

interaction (Figures 4C and 4D). The proximity of the slopes to unity (0.90, 0.88) and superiority of this description over that in terms of co-operative binding (by *F*-test comparison) indicates that the interaction between folate and FBP is also essentially stoichiometric at neutral pH.

Further details of the situation is provided by re-examination in stoichiometric format of equilibrium dialysis data reported by Hansen et al. [3] for a range of FBP concentrations. Whereas the plot for the lowest FBP concentration (0.23 nM) is curvilinear (Figure 5A), the forms of those for 8.2 nM (Figure 5B) and 2.2 μ M (Figure 5C) bear a close resemblance to the linear dependencies observed in the present study for 0.77 and 66 μ M FBP concentrations (Figures 4C and 4D). In this regard, it is significant that the equilibrium results for the lowest FBP concentration were reported to be described well by a rectangular hyperbolic dependence on free folate concentration (Hill coefficient $n = 1.0$) and a dissociation constant K_d of 0.02 nM (see Table 1 in [3]). On the other hand, the corresponding dependencies for results

obtained at the two higher FBP concentrations were sigmoidal ($n \approx 1.4$). It was therefore of interest to determine whether the results presented in the current format (Figures 5A–5C) can be described by the non-co-operative model with the K_d of 0.02 nM, which was deduced from the equilibrium dialysis study at the lowest FBP concentration.

Theoretical dependencies of bound folate concentration, $[folate]_{bound}$, on its total counterpart, $[folate]_{total}$, were calculated from the expressions

$$[folate]_{bound} = \frac{[folate]_{free}[FBP]_{total}}{K_d + [folate]_{free}}$$

$$[folate]_{total} = [folate]_{bound} + [folate]_{free}$$

Specifically, the bound folate concentrations corresponding to assigned values of free folate concentration $[folate]_{free}$ were first calculated for each of the three total acceptor concentrations $[FBP]_{total}$, relevant to Figure 5, after which the total ligand concentration $[folate]_{total}$ was calculated as the sum of free and bound concentrations. As required, there is good conformity in the experimental (closed circle) and theoretical (solid line) dependencies of $[folate]_{bound}$ on the total folate concentration in Figure 5(A). Of greater interest in the present context is the finding that the same model of the folate-FBP interaction also provides very good descriptions of the experimental results obtained with higher FBP concentrations (Figures 5B and 5C). The inability to obtain a satisfactory characterization of folate binding from dependencies of $[folate]_{bound}$ on $[folate]_{free}$ is now seen to reflect the essentially stoichiometric nature of the interaction in mixtures with higher FBP concentration. In that regard, the binding of folate by FBP has been recognized by the Danish group [13] as a stoichiometric interaction in studies of the reaction by quenching of FBP fluorescence.

Whereas a potential source of error in the current estimates of free folate concentration by ultrafiltration of FBP-folate mixtures is rationalized fairly readily, the origin of an overestimate of $[folate]_{free}$ by equilibrium dialysis is less obvious. Clearly, the much lower molecular mass limit for the permeability of dialysis membranes (approx. 2 kDa) renders unlikely any protein contamination of the free-ligand phase. Any overestimation of $[folate]_{free}$ would therefore seem to implicate either preferential binding of unlabelled ligand (thereby increasing the specific activity of free folate) or non-attainment of dialysis equilibrium between the protein-containing (α) and free-ligand (β) phases, which initially comprised folate-free FBP and FBP-free folate respectively. From a theoretical viewpoint, such an explanation is certainly plausible inasmuch as the distribution of free folate (in the absence of FBP) on time t is given by the expression

$$[folate]_t^\beta = [folate]_t^\alpha + [folate]_{t=0}^\beta \exp(-\varepsilon Dt)$$

where D is the translational diffusion coefficient of folate and ε is a cell constant that is dependent on the thickness and effective pore area of the membrane as well as on the volumes of the two phases [31]. Hence, throughout dialysis, the concentration of folate in the ligand-loaded phase (β) exceeds that in the other phase until the passage of sufficient time to render effectively the exponential term to 0. Checks were certainly made [2,3] to establish a time frame for the attainment of dialysis equilibrium (20 h at 37 °C; 24 h at 20 °C). However, any decision about the time independence of $[folate]^\beta$ is a subjective judgement that relies heavily on the sensitivity of the folate assay and the patience of the experimenter. Furthermore, the time required to attain dialysis equilibrium increases markedly with decreasing concentrations of

acceptor (FBP) and ligand (folate) owing to (i) the lower rate of complex formation and (ii) the lower rate of ligand diffusion across the dialysis membrane in response to the smaller gradient in free folate concentration.

Lack of equilibrium attainment was found to have little effect on the dependence of $[\text{folate}]_{\text{bound}}$ on $[\text{folate}]_{\text{total}}$ (Figures 3–5) since the calculation of the former as the difference between $[\text{folate}]_{\text{total}}$ and $[\text{folate}]_{\text{free}}$ still provides a reasonable estimation of bound folate in situations where $[\text{folate}]_{\text{total}} \gg [\text{folate}]_{\text{free}}$. Consequently, the plots of $[\text{folate}]_{\text{bound}}$ versus $[\text{folate}]_{\text{total}}$ in Figures 5(B) and 5(C) identify the folate–FBP interaction at neutral pH to be essentially stoichiometric for the relevant FBP concentrations (8.2 nM and 2.2 μM). Therefore the evidence purporting to establish co-operativity of the interaction between folate and high concentrations of FBP [2,3] can be dismissed since the characterization was attempted under conditions commensurate with an essentially stoichiometric interaction between the reactants. In this regard, the dissociation constant value of 0.02 nM obtained [3] with 0.23 nM FBP is based on very little definitive experimental data. This is evident in Figure 5(A) from a comparison of the curvilinear dependence predicted for that system (solid line) with the linear dependence indicative of stoichiometric interaction (broken line): most of the data are represented adequately by either description. Based on the fact that reliable characterization of an interaction requires the selection of an acceptor concentration such that $[\text{FBP}]_{\text{total}}/K_d < 10$ [32], the lowest FBP concentration (0.23 nM) used by Hansen et al. [3] is the upper limit for an interaction governed by a 20 pM dissociation constant. Indeed, accurate determination of the K_d value for the FBP–folate system at neutral pH requires that experiments are performed with still lower FBP concentrations such that $[\text{FBP}]_{\text{total}}/K_d \approx 1$ [32]. Thus the FBP concentration should be 20 pM (for a system with $K_d = 20$ pM) or even lower in the event that the value obtained by Hansen et al. [3] should prove to be an overestimate of the dissociation constant for the FBP–folate interaction at neutral pH.

It is pertinent at this stage to consider the implications of the present findings to the binding of other folates such as 5-methyltetrahydrofolate, the physiological ligand in milk and blood plasma. FBP is reported to exhibit comparable affinities both for its physiological ligand and for folic acid [2] (e.g. $K_d = 5$ and 3 μM respectively when FBP concentration is 0.1 μM), but those results also showed the dependence of K_d on FBP concentration, identified here as a consequence of stoichiometric binding. Therefore it is probable that the interaction of the physiological ligand with FBP was also essentially stoichiometric under the conditions examined [2]. In addition, it should be pointed out that the present findings imply no criticism of the use of FBP in clinical assays of blood folate concentration, precisely because those assays depend on stoichiometric binding.

Concluding remarks

The purpose of this investigation has been to identify the source of the co-operativity observed [2,3,6] for the interaction between folate and FBP. To that end, our initial studies were undertaken to characterize the interaction under conditions (pH 5.0) where sigmoidal binding behaviour was a theoretical impossibility. Our observation of the same symptoms of co-operativity under these conditions (Figure 2) forced us to question the reliability of the experimental information (albeit reproducible) on which the analysis was based. This doubt led to the subsequent demonstration (Figure 3) that the experiments were essentially stoichiometric titrations. Consideration of our results and also published data [3] at neutral pH (Figures 4 and 5 respectively) has revealed that the symptoms of co-operative binding behaviour under these

conditions were also obtained from data reflecting essentially stoichiometric interaction between folate and FBP.

Performing binding experiments under those conditions offers an excellent means of defining the reaction stoichiometry, but provides little (if any) meaningful information about the strength of the interaction. Indeed, stoichiometric titrations form an integral part of ligand-binding studies by spectral methods, where knowledge of the reaction stoichiometry is regarded as a pre-requisite for the evaluation of a dissociation constant (K_d) from results obtained under non-stoichiometric conditions (see chapter 3 in [32]). On the other hand, studies of ligand binding by equilibrium dialysis and ultrafiltration methods traditionally entail the simultaneous evaluation of K_d and the reaction stoichiometry from binding data obtained under conditions where there is equilibrium coexistence of the ligand in bound and free states. Therefore published equilibrium dialysis data for the folate–FBP system [2,3,6] were analysed on the seemingly reasonable basis that the presence of folate in the non-protein phase (β) had precluded the possibility of stoichiometric complex formation, for which $[\text{folate}]^\beta$ should be zero at equilibrium in experiments with $[\text{FBP}]_{\text{total}} > [\text{folate}]_{\text{total}}$. By demonstrating a flaw in that logic, the present study has emphasized the importance of subjecting binding data to stoichiometric analysis in instances where classical interpretation signifies their incompatibility with theoretical predictions for any physically realistic model of the system. Thus a solution has at last been found to a dilemma that has pervaded the folate–FBP literature for more than 20 years.

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