Cooperativity in Ligand Binding to Dihydrofolate Reductase†

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ABSTRACT: 2,4-Diaminopyrimidine and *p*-aminobenzoyl-L-glutamate can be regarded as "fragments" of methotrexate, a potent inhibitor of dihydrofolate reductase. The equilibrium constants for the binding of these "fragments" and of a series of structurally related compounds to *Lactobacillus casei* dihydrofolate reductase have been determined by fluorimetric methods. 2,4-Diaminopyrimidine and *p*-aminobenzoyl-L-glutamate bind *cooperatively* to the enzyme, in the sense that *p*-aminobenzoyl-L-glutamate binds 54-fold more tightly to the enzyme–2,4-diaminopyrimidine complex than to the enzyme alone. The reciprocal nature of this effect has been quantitatively confirmed. Alkyl substitution on the amino nitrogen of *p*-aminobenzoyl-L-glutamate leads to a marked (up to 130-fold) increase in the affinity for the enzyme alone, but is almost without effect on the affinity for the enzyme–2,4-diaminopyrimidine complex. A similar pattern of behavior is observed with 5- and/or 6-substituted 2,4-diaminopyrimidines. The presence of the other "fragment" thus not only alters the affinity, but significantly alters the specificity of the binding site. For large alkyl substituents (particularly when these are present on both "fragments"), negative cooperativity is observed. The coenzyme, NADPH, also affects the binding of these ligands; it increases the binding constants of both 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate without substantially altering the cooperativity between them. Again, there is a change in specificity for the "fragments" produced by NADPH binding.

Dihydrofolate reductase is the target for the important "anti-folate" drugs such as methotrexate and trimethoprim (Hitchings & Burchall, 1965; Blakley, 1969). Methotrexate is a powerful inhibitor of this enzyme, binding up to 10 000 times more tightly than the structurally very similar substrate, folate. One of our goals in studying this enzyme is to establish the structural basis of this striking difference in affinity (Roberts, 1977; Birdsall et al., 1977; Feeney et al., 1977; Hood & Roberts, 1978; Roberts et al., 1977). In the course of our studies of ligand binding to *Lactobacillus casei* dihydrofolate reductase by high-resolution NMR spectroscopy, we examined the binding of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate, which can be regarded as "fragments" of methotrexate. These two compounds were found to bind to the enzyme not only simultaneously but *cooperatively*, in that *p*-aminobenzoyl-L-glutamate binds substantially more tightly to the enzyme–2,4-diaminopyrimidine complex than to the enzyme alone (Birdsall et al., 1977). We now report further studies of the binding of these and related compounds to dihydrofolate reductase which confirm the reciprocal nature of the interaction between the two ligands, reveal the existence of both positive and negative cooperativity in ligand binding to this enzyme, and show that the specificity of ligand binding is modulated in an important way by the presence of other ligands.

Experimental Section

Materials. Dihydrofolate reductase was isolated and purified from *L. casei* MTX/R as described by Dann et al. (1976); its concentration was determined from its absorbance at 280 nm, by assaying its catalytic activity, and by fluorimetric titration with methotrexate (Dann et al., 1976). 2,4-Diaminopyrimidine (Bachem, Inc.), *p*-aminobenzoyl-L-glutamate (Sigma Chemical Co.), and 2,4,6-triaminopyrimidine (Aldrich Chemical Co.) were recrystallized from water before use. 2,4-Diamino-5,6-dimethylpyrimidine, 2,4-diamino-5-propyl-6-methylpyrimidine, and 2,4-diamino-5-phenoxypyrimidine were generous gifts from Dr. J. J. Burchall, Burroughs Wellcome Research Laboratories, Research Triangle Park, N.C. All other compounds were of the highest purity commercially available and were used without further purification.

Synthesis of Analogues of p-Aminobenzoyl-L-glutamate. *N*-(*p*-Alkylaminobenzoyl)-L-glutamate derivatives were prepared by a modification of the method of Borch & Hassid (1972). *N*-(*p*-Aminobenzoyl)-L-glutamate (2 mmol) was dissolved in CH_3CN/EtOH, 1:1 (100 mL) and the appropriate aldehyde (2 mmol) added. After 20 min, sodium cyanoborohydride (2 mmol) was added and the solution stirred for 10 min at room temperature. Thin-layer chromatography (silica gel; benzene/ethanol/acetic acid, 75:15:15) showed a mixture of the starting material, mono-*N*-alkyl and di-*N*-alkyl derivatives. After acidification with acetic acid, the solution was evaporated to dryness under reduced pressure and the solid was dissolved in benzene/ethanol/acetic acid (80:10:2), applied to a silica gel column, and eluted with the same solvent. The di-*N*-alkyl derivative was eluted first, followed by the mono-*N*-alkyl derivative. In general the chromatographic procedure had to be repeated to obtain mono-*N*-alkyl derivatives which were pure as judged by thin-layer chromatography. Fractions containing pure mono-*N*-alkyl compound were pooled, evaporated to dryness, and dried in vacuo over KOH. The resulting gum or amorphous deliquescent solid was dissolved in water, neutralized to pH 6.8 with NaOH solution, and lyophilized to give a white powder of the disodium salt of the *N*-(*p*-alkylaminobenzoyl)-L-glutamate. This was pure as judged by thin-layer chromatography and NMR spectroscopy and gave a satisfactory elemental analysis. Yields varied between 10 and 35%. *N*-(*p*-Diethylaminobenzoyl)-L-glutamate was prepared and purified in the same way, except that excess aldehyde (5 equiv) was used in the reaction.

For the synthesis of *N*-(*p*-dimethylaminobenzoyl)-L-glu-

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tamate, *p*-dimethylaminobenzoyl chloride (prepared by treatment of *p*-dimethylaminobenzoic acid with thionyl chloride in benzene, followed by removal of solvent and excess thionyl chloride under reduced pressure) was added to a solution of diethyl glutamate in dichloromethane. To this solution was added $KHCO_3$ in water, and the mixture was thoroughly agitated for 2 h. The organic phase was separated, washed, and dried over sodium sulfate. The product was purified by silica gel chromatography, and the diethyl ester then saponified in KOH. The free acid was obtained crystalline (mp 91–92 °C).

N-(*p*-Isopropylbenzoyl)-L-glutamic acid was prepared by acylation of L-glutamic acid with *p*-isopropylbenzoyl chloride in aqueous solution, maintaining the pH at 9.5 by addition of 1 N NaOH, and purified as described above for the *N*-(*p*-alkylaminobenzoyl)-L-glutamates.

For the preparation of *N*-(*p*-*N*′,*N*′,*N*′-trimethylammonium benzoyl)-L-glutamic acid, *p*-dimethylaminobenzoic acid was quaternized with methyl iodide in methanol to give the quaternary methiodide (mp 232–234 °C dec), which was converted to the acid chloride using thionyl chloride. The acid chloride was used to acylate L-glutamic acid in aqueous solution (pH 9.5). After 12 h the solution was acidified with HCl to pH 3 and lyophilized, and the resulting solid was triturated with ethanol. The product was precipitated from the ethanolic solution with ether; repeated precipitations from ethanol gave the pure product (mp 190–193 °C dec).

All compounds were pure as judged by thin-layer chromatography and NMR spectroscopy and gave elemental analyses and NMR and UV spectra in agreement with the proposed structure.

Methods. The binding of *p*-aminobenzoyl-L-glutamate and related compounds was followed by measuring the increase in ligand fluorescence at 350–360 nm on binding. Excitation wavelengths in the long-wavelength edge of the absorption band of the ligand (310–340 nm) were used to minimize self-absorption and the contribution from protein fluorescence. The binding of 2,4-diaminopyrimidine and related compounds leads to a quenching of the protein fluorescence (excitation 295 nm, emission 330 nm) and was followed in this way; for the ternary complexes, the quenching of the fluorescence of bound *p*-aminobenzoyl-L-glutamate was used instead. Ligand binding to the enzyme–NADPH complex was followed by measuring changes in the fluorescence of the bound coenzyme at ~440 nm.

All measurements were made at 25 °C, in a buffer of 15 mM Bistris [bis(2-hydroxyethyl)aminotris(hydroxymethyl)-methane] chloride, pH 6.0, containing 0.5 M KCl. Depending on the magnitude of the binding constant to be determined, enzyme concentrations of 5–500 μM were used; no dependence of binding constant on enzyme concentration was observed. Ligands were added as microliter volumes of concentrated stock solutions. A Perkin-Elmer spectrofluorimeter was used, with 1-cm pathlength quartz cells. Appropriate controls were used to determine the amount of self-absorption and the fluorescence of the free ligand.

Data Analysis. The fluorescence measurements amount to a measurement of the fractional saturation of the enzyme with ligand at a particular ligand concentration. In the case where the enzyme fluorescence is monitored

$$\frac{[EL]}{E_T} = \frac{F_0 - F}{F_0 - F_\infty} \tag{1}$$

where [EL] is the concentration of the complex at a ligand concentration giving a fluorescence intensity F, E_T is the total enzyme concentration, and F_0 and F_∞ are the fluorescence intensities at zero and at saturating ligand concentration, respectively. Similarly, when the fluorescence of the ligand is monitored

$$\frac{[EL]}{E_T} = \frac{F - F_L}{F_\infty - F_L} \tag{2}$$

where F_L is the fluorescence of an equal concentration of ligand in the absence of enzyme. In addition, from the mass action equation

$$K' = [EL]/[E][L]$$

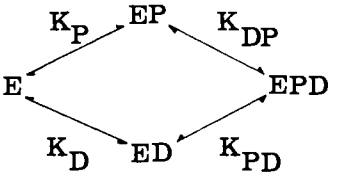
we have

$$\frac{[EL]}{E_T} = \frac{1}{2E_T}\left\{\left(\frac{1}{K'} + E_T + L_T\right) - \left[\left(\frac{1}{K'} + E_T + L_T\right)^2 - 4E_TL_T\right]^{1/2}\right\} \tag{3}$$

where K' is the binding constant and L_T is the total ligand concentration. Combining eq 3 with either eq 1 or 2, the change in fluorescence as a function of ligand concentration can be described in terms of two unknowns, K' and F_∞. These were estimated by a nonlinear least-squares fit of at least 15 values of (F,L_T) to the above equations, incorporating corrections for dilution and, where necessary, for self-absorption.

Thermodynamic Relationships. The equilibria describing the binding of *p*-aminobenzoyl-L-glutamate (P) and 2,4-diaminopyrimidine (D) to the enzyme are shown in Scheme I.

SCHEME I: Equilibria Describing the Binding of 2,4-Diaminopyrimidine (D) and *p*-Aminobenzoyl-L-glutamate (P) to Dihydrofolate Reductase (E).



The four equilibrium constants[1] are defined as follows:

$$K_P = [EP]/[E][P];\ K_D = [ED]/[E][D] \tag{4}$$

$$K_{PD} = [EPD]/[ED][P];\ K_{DP} = [EPD]/[EP][D] \tag{5}$$

Since the standard Gibbs free energy change for the process (E + D + P) → EDP must be the same whichever pathway is followed

$$\Delta G^\circ_P + \Delta G^\circ_{DP} = \Delta G^\circ_D + \Delta G^\circ_{PD} \tag{6}$$

(see Weber, 1975), where the Gibbs energy changes are identified by the same subscripts as the equilibrium constants (eq 4 and 5). Therefore

$$K_PK_{DP} = K_DK_{PD} \tag{7}$$

This relationship can also be derived from Wyman's (1948, 1964) theory of linked functions, and from eq 4 and 5 above. The "cooperativity" in binding between two ligands is defined as

$$K_{coop} = \frac{K_{PD}}{K_P} = \frac{K_{DP}}{K_D} \tag{8}$$

and, in terms of Gibbs energy changes

$$\Delta G^\circ_{coop} = \Delta G^\circ_{PD} - \Delta G^\circ_P = \Delta G^\circ_{DP} - \Delta G^\circ_D \tag{9}$$

[1] These are strictly *apparent* equilibrium constants, as they refer only to a single set of solution conditions and do not take account of, for example, the possible involvement of hydrogen ions.

TABLE I: Equilibrium Constants[a] for the Binding of *p*-Aminobenzoyl-L-glutamate and 2,4-Diaminopyrimidine to *L. casei* Dihydrofolate Reductase.

Ligand	Binary complex	Ternary complex	Cooperativity
p-Aminobenzoyl-L-glutamate	$K_P = 0.83 (\pm 0.07) \times 10^3\ M^{-1}$	$K_{PD} = 5.02 (\pm 0.25) \times 10^4\ M^{-1}$	60 (±9)
2,4-Diaminopyrimidine	$K_D = 1.28 (\pm 0.08) \times 10^3\ M^{-1}$	$K_{DP} = 6.02 (\pm 0.25) \times 10^4\ M^{-1}$	47 (±7) (mean 54)

[a] Defined by Scheme 1 and eq 4 and 5. The standard deviations given are those obtained from the nonlinear regression analysis. Replicate determinations indicate that the upper limit to the standard deviation of our estimates of the binding constants (for all compounds unless otherwise noted) is ±15% for the binary complexes and ±20% for the ternary complexes.

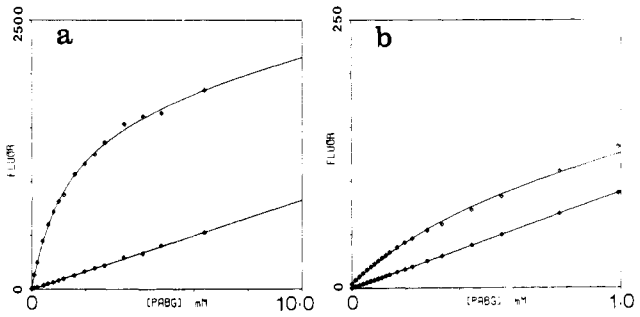


FIGURE 1: Enhancement of the fluorescence of *p*-aminobenzoyl-L-glutamate (PABG) on binding to (a, left) dihydrofolate reductase and (b, right) the dihydrofolate reductase–2,4-diaminopyrimidine complex. In both a and b, the lower line shows the fluorescence of the ligand alone, the upper line that in the presence of enzyme. Points are experimental (after correction for self-asorption) and the lines are best-fit theoretical curves. In both cases the enzyme concentration was 0.1 mM; in b the concentration of 2,4-diaminopyrimidine was 3.2 mM. Note the difference in scale on both abscissa and ordinate between a and b.

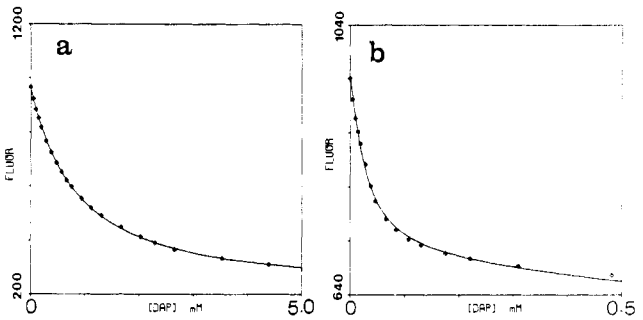


FIGURE 2: Quenching of the fluorescence of (a, left) dihydrofolate reductase and (b, right) the dihydrofolate reductase–*p*-aminobenzoyl-L-glutamate complex on addition of 2,4-diaminopyrimidine (DAP). In a, the fluorescence monitored is that of the enzyme, in b it is that of the bound *p*-aminobenzoyl-L-glutamate. Points are experimental (after correction for self-absorption) and the lines are best-fit theoretical curves. In a the enzyme concentration was 50 μM, in b it was 20 μM; in b, PABG was present at a concentration of 8.1 mM. Note the difference in scale on both abscissa and ordinate between a and b.

where $\Delta G°_{coop}$ is Weber's (1975) "free energy coupling". With these definitions, $K_{coop} > 1$ ($\Delta G°_{coop}$ negative) corresponds to "positive" cooperativity, where the presence of one ligand facilitates the binding of the other, and $K_{coop} < 1$ ($\Delta G°_{coop}$ positive) corresponds to "negative" cooperativity. If $K_{coop} = 1$ ($\Delta G°_{coop} = 0$), the binding of the two ligands is independent. The relationships 8 and 9 define the reciprocal nature of the cooperativity between the two ligands and serve as an important check on the experimental results.

The equilibrium constants K_P and K_D can be determined directly. However, since K_{PD} and K_{DP} refer to the situation where the enzyme is *wholly* saturated with the first ligand, a situation which is not experimentally accessible, the measured binding constant, K_{app}, for P in the presence of a fixed concentration of D must be corrected for the incomplete saturation with D in order to obtain the true equilibrium constant K_{PD}. The relevant equation is

$$K_{PD} = \frac{K_{app}(1 + K_D[D]) - K_P}{K_D[D]} \qquad (10)$$

where [D] is the concentration of *free* D; this was approximated by $(D_T - E_T)$. Comparison of the values of K_{PD} obtained from experiments at various values of D_T (see below) showed that this approximation was valid (to within the error in measuring the binding constants) for $K_D[D] > 3$, the range of values used in practice. The same considerations clearly apply to the estimation of K_{DP}.

Results

The binding of *p*-aminobenzoyl-L-glutamate to *L. casei* dihydrofolate reductase is accompanied by a marked (more than 100-fold) increase in the fluorescence of the ligand at 350 nm, providing a convenient method of determining its binding constant. The binding curve is shown in Figure 1, together with that for *p*-aminobenzoyl-L-glutamate binding to the enzyme in the presence of 2,4-diaminopyrimidine. It is apparent that the presence of the latter compound significantly increases the binding constant of *p*-aminobenzoyl-L-glutamate. The binding of 2,4-diaminopyrimidine leads to quenching of the enzyme fluorescence or, in the ternary complex, to the quenching of the fluorescence of *p*-aminobenzoyl-L-glutamate. The binding curves obtained are shown in Figure 2, from which it is clear that *p*-aminobenzoyl-L-glutamate has the anticipated reciprocal effect of increasing the affinity of the enzyme for 2,4-diaminopyrimidine.

The binding constants obtained in this way are presented in Table I. Since we have been able to measure all four equilibrium constants in Scheme I, we have two independent estimates of K_{coop} and $\Delta G°_{coop}$. These agree satisfactorily, being within 13% of the mean value, so for *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine we have $K_{coop} = 54 (\pm 7)$ and $\Delta G°_{coop} = -2.35 (\pm 0.7)$ kcal/mol, a substantial degree of cooperativity.

N-Alkyl Derivatives of p-Aminobenzoyl-L-glutamate. In order to explore the way in which this cooperativity depends upon the structure of the ligands, we have prepared a series of *N*-alkyl substituted derivatives of *p*-aminobenzoyl-L-glutamate and studied their binding to the enzyme alone and in the presence of 2,4-diaminopyrimidine. The measured binding constants[2] are given in Table II, and the results are shown in

[2] For most of these compounds we have again been able to confirm the reciprocal nature of the cooperativity by measuring all four equilibrium constants in Scheme I. This shows that relationships 8 and 9 do indeed hold in this system, so that, in those cases (here and in subsequent tables) where only three of the four equilibrium constants have been determined experimentally, we can confidently calculate the fourth from eq 8 (or equivalently eq 7).

TABLE II: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of *N*-Alkyl *p*-Aminobenzoyl-L-glutamates to *L. casei* Dihydrofolate Reductase in the Presence and Absence of 2,4-Diaminopyrimidine.

N substituent	K_P (M^{-1})	$\Delta G°_P$ (kcal/mol)	K_{PD} (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{DP}[b] (M^{-1})	$\Delta G°_{DP}$ (kcal/mol)	K_{coop}	$\Delta G°_{coop}$ (kcal/mol)
H	0.83×10^3	−3.97	5.02×10^4	−6.39	6.02×10^4	−6.49	54	−2.35
Methyl	1.05×10^3	−4.10	3.97×10^4	−6.25	2.95×10^4	−6.07	30	−2.00
Ethyl	2.81×10^4	−6.04	(7.31×10^4)	(−6.61)[c]	3.28×10^3	−4.78	2.6	−0.6
Propyl	3.23×10^4	−6.13	4.17×10^4	−6.28	1.51×10^3	−4.32	1.2	−0.12
Butyl	4.74×10^4	−6.35	(4.52×10^4)	(−6.32)[c]	1.22×10^3	−4.20	0.95	0
Pentyl	6.14×10^4	−6.50	4.84×10^4	−6.36	0.91×10^3	−4.02	0.75	+0.17
Hexyl	1.08×10^5	−6.84	(4.79×10^4)	(−6.36)[c]	0.57×10^3	−3.74	0.45	+0.48

[a] Defined by eq 4 and 5. [b] $K_D = 1.28 \times 10^3$ M^{-1}; $\Delta G°_D = -4.22$ kcal/mol. [c] Values in parentheses were not determined directly, but calculated from measured values of K_P, K_D, and K_{DP} using eq 7.

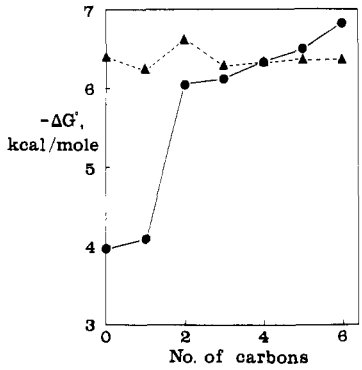


FIGURE 3: Change in Gibbs energy on binding of *N*-alkyl-*p*-aminobenzoyl-L-glutamates to dihydrofolate reductase, alone (●—●) or in the presence of 2,4-diaminopyrimidine (▲- - -▲), as a function of the number of carbon atoms in the *N*-alkyl group.

Figure 3 in the form of a plot of $-\Delta G°$ against the number of carbons in the alkyl chain.

N-Alkyl substitution clearly leads to a significant increase in affinity for the enzyme alone; *N*-hexyl *p*-aminobenzoyl-L-glutamate binds 130 times more tightly than does *p*-aminobenzoyl-L-glutamate itself. The major part of this increase, some 27-fold, occurs on going from the *N*-methyl to the *N*-ethyl derivative, followed by only a modest further increase as the alkyl chain is lengthened further. Although the *N*-alkyl substituent has a substantial effect on the formation of the binary complex, it has little effect on the binding of these compounds to the enzyme–2,4-diaminopyrimidine complex. The equilibrium constant K_{PD} is close to 5.0×10^4 M^{-1} for all the compounds in Table II, having a range of only 1.8-fold compared with the 130-fold range of K_P. It is clear that the presence of 2,4-diaminopyrimidine substantially alters the way in which the alkyl chains interact with the enzyme. The cooperativity between 2,4-diaminopyrimidine and the *p*-aminobenzoyl-L-glutamates thus varies substantially with alkyl chain length, from positive cooperativity for *p*-aminobenzoyl-L-glutamate itself and for its *N*-methyl derivative (K_{coop} = 54 and 30, respectively), through essentially independent binding ($K_{coop} \sim 1$) for the *N*-ethyl to *N*-pentyl derivatives, to significant negative cooperativity (K_{coop} = 0.45) for the *N*-hexyl compound.

Other Analogues of p-Aminobenzoyl-L-glutamate. Table III shows the results for some other analogues of *p*-aminobenzoyl-L-glutamate. *p*-(*N*,*N*-Dimethylamino)-benzoyl-L-glutamate has a binding constant and a degree of cooperativity intermediate between those of the *N*-methyl and *N*-ethyl compounds, while the *N*,*N*-diethyl compound shows behavior similar to that of *N*-butyl *p*-aminobenzoyl-L-glutamate. Comparison of the *N*,*N*-dimethylamino compound with *p*-isopropylbenzoyl-L-glutamate suggests that the amino nitrogen atom is not a major determinant of the binding or of the cooperativity. The quaternary ammonium compound, *p*-(*N*,*N*,*N*-trimethylamino)benzoyl-L-glutamate, binds very weakly to the enzyme but still shows cooperativity comparable to that seen with the *N*,*N*-dimethylamino compound.

Analogues of 2,4-Diaminopyrimidine. A limited number of analogues of diaminopyrimidine have been studied; the results are presented in Tables IV and V. Substitution of an amino group at the 6-position of 2,4-diaminopyrimidine has little effect on either the binding or the cooperativity (compare Table I). However, alkyl or aryl substitution at the 5- and/or 6-position markedly increases the equilibrium constant for formation of the binary complex (Table IV). (The effect of 5-aryl groups is of course well known (Hitchings & Burchall, 1965).) Trimethoprim binds to *L. casei* dihydrofolate reductase with an equilibrium constant of 1.7×10^7 M^{-1} at pH 6.5 (Hood & Roberts, 1978).) The binding constants for the compounds shown in Table IV range up to 390 times that of 2,4-diaminopyrimidine, but the binding constants for these compounds to the enzyme–*p*-aminobenzoyl-L-glutamate complex cover a much smaller range—only 4.5-fold.

The effects of 2,4-diamino-5-propyl-6-methylpyrimidine on the binding of some *N*-alkyl derivatives of *p*-aminobenzoyl-L-glutamate are shown in Table V. It was noted above that the *N*-alkyl substituents had virtually no effect on binding to the enzyme–2,4-diaminopyrimidine complex. There is some effect of the alkyl group on binding to the enzyme–2,4-diamino-5-propyl-6-methylpyrimidine complex, as the binding constants of *p*-aminobenzoyl-L-glutamate and its *N*-pentyl derivative differ by 5-fold, but this is again much less than the 74-fold difference in the equilibrium constants for formation of the binary complexes. The values of K_{coop} observed in this situation are uniformly substantially lower than those found with 2,4-diaminopyrimidine; in particular the *N*-propyl and *N*-pentyl *p*-aminobenzoyl-L-glutamates bind only one-fifth as well to the enzyme–2,4-diamino-5-propyl-6-methylpyrimidine complex as to the enzyme alone. Clearly it is important to establish that this is indeed negative cooperativity and not simply competition. This distinction can readily be made by measuring the apparent binding constant of 2,4-diamino-5-propyl-6-methylpyrimidine in the presence of various concentrations of *N*-pentyl *p*-aminobenzoyl-*L*-glutamate. For simple competition

$$K_{app} = \frac{K_D}{1 + K_P[P]} \tag{11}$$

while for negative cooperativity

TABLE III: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of Analogues of *p*-Aminobenzoyl-L-glutamate to *L. casei* Dihydrofolate Reductase in the Presence and Absence of 2,4-Diaminopyrimidine.

Compound	K_P (M^{-1})	$\Delta G°_P$ (kcal/mol)	K_{PD} (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{DP}[b] (M^{-1})	$\Delta G°_{DP}$ (kcal/mol)	K_{coop}	$\Delta G°_{coop}$ (kcal/mol)
p-(*N*,*N*-Dimethylamino)benzoyl-L-glutamate	7.94×10^3	−5.30	1.60×10^5	−7.07	1.27×10^4	−5.58	15	−1.57
p-(*N*,*N*-Diethylamino)benzoyl-L-glutamate	6.72×10^4	−6.56	8.12×10^4	−6.67	1.16×10^3	−4.16	1.1	−0.06
p-Isopropylbenzoyl-L-glutamate	2.29×10^3	−4.56	(2.0×10^4)	(−5.85)[c]	1.13×10^4	−5.50	9	−1.29
p-(*N*,*N*,*N*-Trimethylamino)-benzoyl-L-glutamate	76	−2.55	(610)	(−3.78)[c]	1.03×10^4	−5.45	8	−1.23

[a] Defined in eq 4 and 5. [b] $K_D = 1.28 \times 10^3$ M^{-1}; $\Delta G°_D = -4.22$ kcal/mol. [c] Values in parentheses were not determined directly but calculated from measured values of K_P, K_D, and K_{DP} and eq 7.

TABLE IV: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of Substituted 2,4-Diaminopyrimidines to *L. casei* Dihydrofolate Reductase, in the Presence and Absence of *p*-Aminobenzoyl-L-glutamate.

Substituent(s)	K_D (M^{-1})	$\Delta G°_D$ (kcal/mol)	K_{DP} (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{PD}[b] (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{coop}	$\Delta G°_{coop}$ (kcal/mol)
6-NH_2	1.64×10^3	−4.37	(6.4×10^4)	(−6.53)[c]	3.2×10^4	−6.12	39	−2.15
5,6-Me_2	2.43×10^4	−5.96	(1.2×10^5)	(−6.90)[c]	4.07×10^3	−4.90	4.9	−0.93
5-Pr,6-Me	8.13×10^4	−6.67	(2.68×10^5)	(−7.37)[c]	2.73×10^3	−4.67	3.3	−0.70
5-Phenoxy	5.0×10^5	−7.74	(2.1×10^5)	(−7.23)[c]	3.5×10^2	−3.46	0.4	+0.51

[a] Defined in eq 4 and 5. [b] $K_P = 0.83 \times 10^3$ M^{-1}; $\Delta G°_P = -3.97$ kcal/mol. [c] Values in parentheses were not determined directly, but calculated from measured values of K_D, K_P, and K_{PD} and eq 7.

TABLE V: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of *N*-Alkyl *p*-Aminobenzoyl-L-glutamates to *L. casei* Dihydrofolate Reductase, in the Presence and Absence of 2,4-Diamino-5-propyl-6-methylpyrimidine.

N substituent	K_P (M^{-1})	$\Delta G°_P$ (kcal/mol)	K_{PD} (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{DP}[b] (M^{-1})	$\Delta G°_{DP}$ (kcal/mol)	K_{coop}	$\Delta G°_{coop}$ (kcal/mol)
H	0.83×10^3	−3.97	2.73×10^3	−4.67	(2.67×10^5)	(−7.37)[c]	3.3	−0.70
Methyl	1.05×10^3	−4.10	(1.63×10^3)	(−4.36)[c]	1.26×10^5	−6.93	1.6	−0.26
Propyl	3.23×10^4	−6.13	(7.11×10^3)	(−5.23)[c]	1.80×10^4	−5.78	0.2	+0.88
Pentyl	6.14×10^4	−6.50	(1.38×10^4)	(−5.62)[c]	1.83×10^4	−5.79	0.2	+0.88

[a] Defined in eq 4 and 5. [b] $K_D = 8.13 \times 10^4$ M^{-1}; $\Delta G°_D = -6.67$ kcal/mol. [c] Values in parentheses were not determined directly, but calculated from the other three equilibrium constants and eq 7.

$$K_{app} = \frac{K_D + K_{DP}K_P[P]}{1 + K_P[P]} \qquad (12)$$

where [P] is the concentration of *N*-pentyl *p*-aminobenzoyl-L-glutamate, and the equilibrium constants are defined in Scheme I and eq 4 and 5. In the case of competition, K_{app} of course decreases indefinitely as [P] is increased, and its value at any point can be calculated, using eq 11, from the independently measured values of K_D and K_P. For negative cooperativity on the other hand, K_{app} asymptotes to a finite value, K_{DP}, as [P] increases (eq 12). The data shown in Figure 4 clearly demonstrate that the effect of *N*-pentyl *p*-aminobenzoyl-L-glutamate on the binding of 2,4-diamino-5-propyl-6-methylpyrimidine arises from negative cooperativity, *not* competition.

Ligand Binding to the Enzyme–NADPH Complex. The binding constant of NADPH under the conditions used for these experiments is 1.1×10^8 M^{-1} (S. Dunn & R. W. King, unpublished work), so that effectively complete saturation of the enzyme with coenzyme could be maintained, at the enzyme concentrations used, by adding NADPH at concentrations 1.3–1.5 times the enzyme concentration.

The binding constant of *p*-aminobenzoyl-L-glutamate to the enzyme–NADPH complex is 3.75×10^3 M^{-1}, 4.5 times

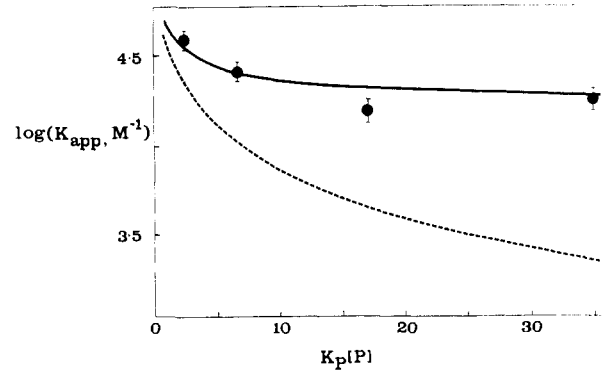


FIGURE 4: The logarithm of the apparent binding constant of 5-propyl-6-methyl-2,4-diaminopyrimidine as a function of the concentration of *N*-pentyl-*p*-aminobenzoyl-L-glutamate (expressed as $K_P[P]$, where K_P is the binding constant for *N*-pentyl-*p*-aminobenzoyl-L-glutamate). Points are experimental; the solid line is calculated using eq 12, cooperativity, with $K_{coop} = 0.2$, while the dashed line is calculated using eq 11, simple competition.

greater than that for binding to the enzyme alone (consistent with the factor of 3.4 obtained earlier (Roberts et al., 1974) by NMR methods under somewhat different conditions). For 2,4-diaminopyrimidine and two 5,6-disubstituted analogues

TABLE VI: Equilibrium Constants and Gibbs Energy Changes for the Binding of 2,4-Diaminopyrimidines to the *L. casei* Dihydrofolate Reductase-NADPH Complex.

Substituent	K_D (M^{-1})	$\Delta G°_D$ (kcal/mol)	K_D(enzyme·NADPH)/K_D(enzyme)
None	1.11×10^4	−5.50	8.7
5,6-Me_2	1.85×10^5	−7.16	7.6
5-Pr,6-Me	6.7×10^5	−7.91	8.2

(Table VI), the increase in binding constant produced by NADPH is a factor of 8 (1.2 kcal/mol).

Binding constants for the *N*-alkyl *p*-aminobenzoyl-L-glutamates to the enzyme-NADPH complex in the presence and absence of 2,4-diaminopyrimidine are given in Table VII. The effect of alkyl chain length on binding to the enzyme-NADPH complex is quite distinct from that seen with the free enzyme. While there is again a marked increase in binding constant (60-fold) on going from *N*-methyl to *N*-ethyl, further lengthening of the chain leads to a *decrease* in binding constant rather than the modest increase noted above for the free enzyme.

The binding of the *N*-alkyl *p*-aminobenzoyl-L-glutamates to the enzyme-NADPH-2,4-diaminopyrimidine complex follows the same general trend as that to the enzyme-NADPH complex, except that the binding constant increases only 3-fold (rather than 60-fold) between *N*-methyl and *N*-ethyl. In addition, the binding of the *N,N*-dimethyl compound in the presence of 2,4-diaminopyrimidine is virtually unaffected by NADPH (compare the values in Tables VII and III). The cooperativity between 2,4-diaminopyrimidine and the *N*-alkyl *p*-aminobenzoyl-L-glutamates is not radically affected by the presence of NADPH, though the values of K_{coop} are somewhat lower. In particular the variation of K_{coop} with alkyl chain length is similar to that observed in the absence of coenzyme.

Discussion

The phenomenon of cooperativity or interdependence in the binding of two or more ligands to a protein is of course well known. In a recent discussion of such effects from the thermodynamic point of view, Weber (1975) has noted that rather few examples are known for monomeric proteins, and that the reciprocal nature of the effects has only rarely been demonstrated quantitatively (Benesch et al., 1968; Mildvan, 1972; Kolb & Weber, 1975; Nowak & Lee, 1977). We have shown here that there is a substantial degree of cooperativity, amounting to a Gibbs energy coupling of −2.35 kcal/mol, between 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate binding to *L. casei* dihydrofolate reductase, a monomeric protein of molecular weight 18 000 (Dann et al., 1976). In addition we have been able to confirm quantitatively the reciprocal nature of this cooperativity, both for these ligands and for a number of their structural analogues. 2,4-Diaminopyrimidine and *p*-aminobenzoyl-L-glutamate can be regarded as "fragments" of the powerful inhibitor methotrexate, and there is good evidence that they bind to the enzyme in a way very similar to that in which the corresponding parts of methotrexate itself bind (see below). We are thus dealing with cooperativity between *subsites* of the inhibitor binding site.

Cooperativity between subsites has been observed for the coenzyme binding site of other dehydrogenases; for example, McPherson (1970) noted that binding of nicotinamide mononucleotide to lactate dehydrogenase could be observed only in the presence of AMP.

The Mechanism of Cooperativity. Interdependence in the binding of two ligands to a protein can either arise from a direct interaction between the ligands in the ternary complex, each ligand forming part of the binding site of the other, or it can be an effect transmitted between two spatially separate binding sites by some conformational change in the protein. Thermodynamic measurements of the kind reported here cannot rigorously distinguish between these two mechanisms. However, while positive cooperativity can readily be accommodated by this first kind of mechanism, the observation of *negative* cooperativity is much more readily explained by postulating a significant (though possibly small) conformational difference between the binary and ternary complexes. In addition, there is clear evidence from stopped-flow kinetic studies (R. W. King & J. G. Batchelor, unpublished work) that a conformational change accompanies methotrexate binding to dihydrofolate reductase, and suggestive, though indirect, evidence from NMR studies (Birdsall et al., 1977; Feeney et al., 1977; Roberts et al., 1977) that this is also true of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate binding. We therefore favor the idea that the observed cooperativity arises from a ligand-induced conformational change.

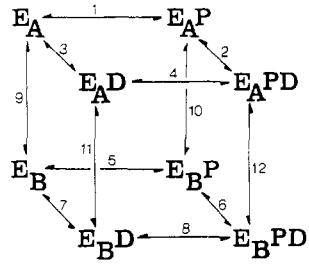
It is convenient to discuss the effects of ligand structure on binding and cooperativity within the framework of a specific model; a simple model involving two conformational states of the enzyme, E_A and E_B, is shown in Scheme II. In this model, positive cooperativity between 2,4-diaminopyrimidine (D) and *p*-aminobenzoyl-L-glutamate (P) will be observed if (i) both ligands bind more tightly to E_B than to E_A, and (ii) K_9 (= $[E_B]/[E_A]$) is small (most probably less than 1). The binding

TABLE VII: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of *N*-Alkyl *p*-Aminobenzoyl-L-glutamates to the *L. casei* Dihydrofolate Reductase-NADPH Complex, in the Presence and Absence of 2,4-Diaminopyrimidine.

N substituent	K_P (M^{-1})	$\Delta G°_P$ (kcal/mol)	K_{PD} (M^{-1})	$\Delta G°_{PD}$ (kcal/mol)	K_{DP}[b] (M^{-1})	$\Delta G°_{DP}$ (kcal/mol)	K_{coop}	$\Delta G°_{coop}$ (kcal/mol)
H	3.75×10^3	−4.85	(9.5×10^4)	(−6.75)[c]	2.81×10^5	−7.40	25	−1.90
Methyl	2.38×10^3	−4.59	(3.3×10^4)	(−6.14)[c]	1.54×10^5	−7.05	14	−1.55
Ethyl	1.48×10^5	−7.02	(1.07×10^5)	(−6.82)[c]	8.0×10^3	−5.30	0.7	+0.20
Propyl	5.88×10^4	−6.48	(9.0×10^4)	(−6.72)[c]	1.69×10^4	−5.74	1.5	−0.24
Butyl	3.45×10^4	−6.16	(2.7×10^4)	(−6.02)[c]	8.8×10^3	−5.36	0.8	+0.14
Pentyl	1.03×10^4	−5.45	(4.7×10^3)	(−4.99)[c]	5.1×10^3	−5.04	0.46	+0.46
Hexyl	3.44×10^4	−6.16	(1.2×10^4)	(−5.54)[c]	3.94×10^3	−4.88	0.35	+0.62
Dimethyl	1.39×10^5	−6.99	(1.55×10^5)	(−7.75)[c]	1.24×10^4	−5.56	1.1	−0.06
Diethyl	2.32×10^5	−7.29	(7.0×10^4)	(−6.58)[c]	3.39×10^3	−4.79	0.3	+0.71

[a] Defined in eq 4 and 5. [b] $K_D = 1.11 \times 10^4\ M^{-1}$; $\Delta G°_D = -5.50$ kcal/mol. [c] Values in parentheses were not determined directly, but were calculated from the measured values of K_P, K_D and K_{DP} using eq 7.

SCHEME II: Equilibria Describing the Binding of 2,4-Diaminopyrimidine (D) and *p*-Aminobenzoyl-L-glutamate (P) to Two Conformational States of Dihydrofolate Reductase (E_A and E_B).[a]



[a] The numerals attached to each equilibrium indicate the numbering scheme for the equilibrium constants. K_1–K_8 are defined as binding constants (e.g., $K_1 = [E_AP]/[E_A][P]$) and K_9–K_{12} are defined as ratios of E_B to E_A (e.g., $K_{11} = [E_BD]/[E_AD]$).

of the first ligand, for example, D, will increase the proportion of E_B (if $K_7 > K_3$, then $K_{11} > K_9$) and hence the binding constant of the second ligand. The four measurable binding constants of Scheme I can be expressed in terms of the equilibrium constants in Scheme II as follows:

$$K_P = \frac{K_1 + K_5K_9}{(1 + K_9)} \quad (13)$$

$$K_D = \frac{K_3 + K_7K_9}{(1 + K_9)} \quad (14)$$

$$K_{PD} = \frac{K_4 + K_8K_{11}}{(1 + K_{11})} \quad (15)$$

$$K_{DP} = \frac{K_2 + K_6K_{10}}{(1 + K_{10})} \quad (16)$$

These expressions can be simplified, at the cost of some loss of generality in the model, by making two further assumptions: (i) form E_A of the free enzyme predominates,[3] i.e., $K_9 \ll 1$, and (ii) the *only* effect of the binding of one ligand on the affinity for the other is that due to the change in the proportions of the two conformations E_A and E_B, i.e., $K_1 = K_4$, $K_2 = K_3$ and $K_5 = K_8$, $K_6 = K_7$. Equations 13 and 15 then simplify to:

$$K_P = K_1 + K_5K_9 \quad (17)$$

$$K_{PD} = \frac{K_1 + K_5K_{11}}{(1 + K_{11})} \quad (18)$$

Structural Effects on Ligand Binding. *N*-alkyl substitution of *p*-aminobenzoyl-L-glutamate markedly increases binding to the free enzyme but has virtually no effect on binding to the enzyme–2,4-diaminopyrimidine complex. 2,4-Diaminopyrimidine thus not only affects the binding constant of the second ligand, but also alters the structural specificity of its binding site, and clearly the interactions which the *N*-alkyl chains make with the enzyme must be different in the binary and ternary complexes. In terms of the model represented by Scheme II and eq 17 and 18, these observations imply that *N*-alkyl substitution of *p*-aminobenzoyl-L-glutamate increases K_1, but has no effect on K_5. The observed value of K_{PD} is approximately 5×10^4 M^{-1} for all the *N*-alkyl *p*-aminobenzoyl-L-glutamates, and this is a lower limit to K_5. From eq 17 and the value of K_P for *p*-aminobenzoyl-L-glutamate of 0.83×10^3 M^{-1}, this limit for K_5 sets an upper limit to K_9 of 0.016, while from eq 18 and K_{PD}, $K_{11} > 10$. Therefore $K_{11}/K_9 \geq 625$, and 2,4-diaminopyrimidine must bind at least 625 times more tightly to E_B than to E_A, a Gibbs energy difference of −3.8 kcal/mol. For *p*-aminobenzoyl-L-glutamate $K_1 \leq 0.8 \times 10^3$ M^{-1}, and as the alkyl chain is lengthened this increases to $\sim 1.0 \times 10^5$ M^{-1} for *N*-hexyl *p*-aminobenzoyl-L-glutamate. For the latter compound $K_1 > K_5$, so that the increase in the proportion of E_B produced by 2,4-diaminopyrimidine is unfavorable to its binding, and *negative* cooperativity is observed.

The most obvious basis for the increase in K_1 with increasing length of the *N*-alkyl substituent is a "hydrophobic" interaction with a nonpolar region of the enzyme surface. The interaction with the second methylene in the chain must be a rather specific one, and the nonpolar region must be limited in extent. This nonpolar region disappears or becomes inaccessible to the *N*-alkyl groups in the E_B conformation, since K_{PD} (hence K_5) is essentially independent of chain length for the *N*-alkyl compounds.

Only limited data are available for the effects of 5- and/or 6-substitution in 2,4-diaminopyrimidine on binding, but these seem to follow the same kind of pattern as that observed for the *N*-alkyl-substituted *p*-aminobenzoyl-L-glutamates, in that they are much larger for the binary than for the ternary complexes. In terms of the model of Scheme II, the results in Table IV can be explained by postulating that increasing the bulk of the substituents on the 2,4-diaminopyrimidine ring markedly increases K_3, but has only a small effect on K_7. These results imply the existence of a binding site for the alkyl or aryl substituents which is in some way more accessible in conformation E_A than in conformation E_B.

Is it possible that this site is the same region of the enzyme surface that is involved in the binding of the *N*-alkyl substituents of the *p*-aminobenzoyl-L-glutamates? If this were the case, then, with sufficiently large substituents on *both* the 2,4-diaminopyrimidine and the *p*-aminobenzoyl-L-glutamate, one would expect at least some interference and perhaps competition in binding to the E_A conformation. Competitive binding would not be observed experimentally, however, since the two ligands should still be able to bind simultaneously to E_B, in which the "alkyl" binding site is apparently unimportant. Such interference in binding would invalidate the simplified version of the model discussed above (as represented by eq 17 and 18), since it would require $K_4 < K_1$. The simplified model is in fact *not* adequate to explain the results for the binding of *N*-alkyl *p*-aminobenzoyl-L-glutamates in the presence of 2,4-diamino-5-propyl-6-methylpyrimidine presented in Table V. According to this model, the only difference between 2,4-diaminopyrimidine itself and the 5-propyl-6-methyl derivative will be in K_3 and K_7, and hence in K_{11}. Using the lower limit to K_5 of 5×10^4 M^{-1} derived above, the low value of K_{coop} observed for *p*-aminobenzoyl-L-glutamate in the presence of the 5-propyl-6-methyl compound requires $K_{11} \sim 0.06$ (this is still larger than $K_9 \leq 0.016$, so positive cooperativity is observed). Now with $K_{11} \ll 1$, the lower limit to K_{PD}, from eq 18, is clearly K_1. For *N*-pentyl *p*-aminobenzoyl-L-glutamate, for example, $K_P = 6 \times 10^4$ M^{-1} and since $K_5K_9 \leq 0.8 \times 10^3$ M^{-1}, then $K_1 \sim 6 \times 10^4$ M^{-1} (eq 17); this is larger than the observed value of K_{PD}, 1.38×10^4 M^{-1}, and in fact for $K_{11} \ll 1$, negative cooperativity should not be observed. In order to explain the results in Table V, one must postulate that, for the longer chain *N*-alkyl *p*-aminobenzoyl-L-glutamates in the presence of 2,4-diamino-5-propyl-6-methylpyrimidine, $K_4 <$

[3] Stopped-flow studies of ligand binding (R. W. King, S. Dunn, & J. G. Batchelor, unpublished work) have shown that, in the absence of ligands, *L. casei* dihydrofolate exists as a mixture of comparable amounts of two interconverting conformational states, E_1 and E_2. These cannot, however, correspond to E_A and E_B of Scheme II, since NADPH binds exclusively to E_1 and yet has only a small effect on the cooperativity between 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate (see below).

K_1 (so that eq 13 and 15 must be used in place of eq 17 and 18). This is therefore consistent with the idea that the binding sites for the alkyl substituents of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate are at least partly overlapping. However, it must be emphasized that, since we can only measure three independent equilibrium constants, no model more elaborate than that of Scheme I can be rigorously justified. Tests of the model shown in Scheme II (and of the predictions made on the basis of this model from the data reported here) will depend upon characterization of the conformational states E_A and E_B; experiments directed toward this end, using fluorescence and NMR spectroscopy, are currently in progress.

Effects of Coenzyme Binding. NADPH is known to increase the binding constants of a number of inhibitors to dihydrofolate reductase (Perkins & Bertino, 1966; Williams et al., 1973; Roberts et al., 1974; A. S. V. Burgen & G. C. K. Roberts, unpublished work), and coenzyme has often been observed to bind cooperatively with substrate analogues to other dehydrogenases (see, for example, Theorell & McKinley-McKee, 1961; Kolb & Weber, 1975). NADPH increases the binding both of *p*-aminobenzoyl-L-glutamate and of 2,4-diaminopyrimidine to *L. casei* dihydrofolate reductase. The cooperativity between these two ligands is decreased by only a factor of two in the presence of NADPH, and the values of K_{coop} as a function of the alkyl chain length in *N*-alkyl *p*-aminobenzoyl-L-glutamates follow a very similar pattern in the presence and absence of NADPH. These observations suggest that NADPH binding does not lead to a marked change in the proportions of E_A and E_B (Scheme II); certainly the increase it produces in the binding constants for *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine cannot be due simply to an increase in the proportion of E_B.

The most striking effect of NADPH in the present context is the change it produces in the structural specificity on *N*-alkyl *p*-aminobenzoyl-L-glutamate binding. A tighter binding of *N*-ethyl than of *N*-methyl *p*-aminobenzoyl-L-glutamate is still found to be a characteristic of the E_A conformation. However, in the presence of NADPH, a decrease in binding energy for *N*-alkyl substituents longer than ethyl seems to be characteristic of both E_A and E_B conformations. The effects of NADPH on the binding of *p*-(*N,N*-dimethylamino)benzoyl-L-glutamate are especially notable. For this compound the equilibrium constant for binding to the enzyme·NADPH complex is 17.5 times that for binding to the enzyme alone, while in the presence of 2,4-diaminopyrimidine coenzyme has no effect on its binding. This leads to a decrease in K_{coop} on addition of NADPH, from 15 to 1.1. Clearly the two kinds of cooperativity (between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine, and between these two ligands and NADPH) are not independent in this instance.

Relationship to Methotrexate Binding. The binding constants of the "fragments" of methotrexate studied here cannot be quantitatively compared with that of methotrexate itself (approximately 2×10^9 M^{-1} under these conditions; calculated from the results of Hood & Roberts, 1978), because of the difficulties in defining the appropriate standard state and in accounting properly for the different entropy changes in the two situations (see, for example, Jencks, 1975). However, the observation of cooperativity in the binding of the "fragments" has important qualitative implications for the understanding of methotrexate binding. There is good evidence from NMR studies (Birdsall et al., 1977; Feeney et al., 1977; Kimber et al., 1977; Roberts et al., 1977) that the "fragments" do bind to the enzyme in a way very similar to that in which methotrexate itself binds. The absence of the pyrazine portion of the pteridine ring of methotrexate does not seem to lead to any large perturbation of the mode of binding of the "fragments". Furthermore, there is indirect evidence (discussed by Feeney et al., 1977; Roberts et al., 1977) that some of the chemical shift changes of amino acid resonances observed when the "fragments" bind are due to conformational changes, perhaps related to the conformational changes responsible for the observed cooperativity. Since the same chemical shift changes are seen on methotrexate binding, the same conformational changes must accompany methotrexate binding. It seems likely, therefore, that the cooperativity described here for the "fragments" also plays a part in methotrexate binding in the form of *intra*molecular cooperativity, particularly if methotrexate binds in a step-wise rather than an "all-or-none" manner (Burgen et al., 1975). Such behavior would clearly have important implications for attempts to describe the binding energy of methotrexate in terms of contributions from its constituent parts.

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Properties of 3-Methyladenine-DNA Glycosylase from *Escherichia coli*†

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ABSTRACT: An *Escherichia coli* enzyme that releases 3-methyladenine and 3-ethyladenine in free form from alkylated DNA has been purified 2800-fold in 7% yield. The enzyme does not liberate several other alkylation products from DNA, including 7-methylguanine, O^6-methylguanine, 7-methyladenine, N^6-methyladenine, 7-ethylguanine, O^6-ethylguanine, and the arylalkylated purine derivatives obtained by treatment of DNA with 7-bromomethyl-12-methylbenz[*a*]anthracene. The reaction of the enzyme with alkylated DNA leads to the introduction of apurinic sites but no chain breaks (less than one incision per ten apurinic sites), and there is no detectable nuclease activity with native DNA, depurinated DNA, ultraviolet-irradiated DNA, or X-irradiated DNA as potential substrates. The enzyme is termed 3-methyladenine-DNA glycosylase. It is a small protein, M_r = 19 000, that does not require divalent metal ions, phosphate, or other cofactors in order to cleave base–sugar bonds in alkylated DNA.

When DNA is exposed to alkylating agents such as dimethyl sulfate or methyl methanesulfonate, either in vivo or in vitro, the two major alkylation products are 7-methylguanine and 3-methyladenine. The more strongly mutagenic and carcinogenic alkylating agents *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produce the same two lesions, but in addition relatively large amounts of O^6-methylguanine and phosphotriesters are formed. Several minor alkylation products, e.g., 7-methyladenine, 3-methylguanine, 3-methylcytosine, and O^4-methylthymine, have also been detected (Lawley, 1974; Strauss et al., 1975). Further, ethylated derivatives analogous to the methylation products are obtained with the alkylating agents diethyl sulfate, ethyl methanesulfonate, or *N*-ethyl-*N*-nitrosourea (Sun and Singer, 1975). The fate in vivo of alkylated residues in DNA varies considerably between different types of lesions. Surprisingly, the major product 7-methylguanine does not seem to be actively excised from alkylated DNA in either *Escherichia coli* or mammalian cells but is slowly lost over a period of days by nonenzymatic hydrolysis (Prakash and Strauss, 1970; Lawley and Orr, 1970). On the other hand, 3-methyladenine residues are rapidly removed in both systems by an enzymatic process (Lawley and Orr, 1970; Lawley and Warren, 1976; Margison and O'Connor, 1973). O^6-Methylguanine is also actively released in *E. coli* and mammalian cells but at a considerably slower rate than 3-methyladenine (Lawley and Orr, 1970; Goth-Goldstein, 1977). Analogous results have been obtained for the removal of ethylated purines from DNA (Lawley and Warren, 1975).

These findings strongly suggest that cells exposed to alkylating agents can repair part of the alkylation damage in their DNA by excision-repair processes, and that repair enzymes exist that selectively act on alkylated DNA. The first evidence for the existence of such repair enzymes was obtained by Strauss and co-workers (Strauss, 1962; Reiter et al., 1967; Strauss and Robbins, 1968), who showed that crude cell extracts from *Bacillus subtilis* and *Micrococcus luteus* catalyze the formation of strand breaks at some, but not all, alkylated sites in methyl methanesulfonate treated DNA. While the results were interpreted to reflect the presence of an endonuclease specific for alkylated DNA, the activity was not purified. Similar results were obtained by Friedberg and Goldthwait (Friedberg and Goldthwait, 1969, Friedberg et al., 1969) with a partly purified enzyme fraction from *E. coli* termed endonuclease II. Papirmeister et al. (1970) demonstrated in this connection that *E. coli* cell extracts incise alkylated DNA at 3-methyladenine residues but not at 7-methylguanine residues, in good agreement with the data of Lawley and Orr (1970) on the relative excision rates of different alkylated purines in vivo.

The major component in early preparations of *E. coli* endonuclease II was found to be an endonuclease activity specific for apurinic sites in DNA, apparently due to an endonucleolytic function of exonuclease III, but this enzyme does not act as an endonuclease at alkylated residues in DNA (Hadi and Goldthwait, 1971; Verly et al., 1973; Weiss, 1976; Kirtikar et al., 1976; Ljungquist and Lindahl, 1977; Gossard and Verly, 1978). The endonuclease II preparations investigated by Goldthwait and co-workers have contained several additional enzymatic activities besides the endonuclease activity for apurinic sites (Kirtikar and Goldthwait, 1974; Kirtikar et al., 1975a,b, 1976), and endonuclease II was therefore recently

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