Effects of Coenzyme Analogues on the Binding of *p*-Aminobenzoyl-L-glutamate and 2,4-Diaminopyrimidine to *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The binding of *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine to dehydrofolate reductase from *Lactobacillus casei* MTX/R in the presence of a series of coenzymes and coenzyme analogues has been measured fluorometrically. These two ligands, which can be regarded as "fragments" of the powerful inhibitor methotrexate, have been shown to bind cooperatively in the absence of coenzyme [Birdsall, B., Burgen, A. S. V., Rodrigues de Miranda, J., & Roberts, G. C. K. (1978) *Biochemistry 17*, 2102], *p*-aminobenzoyl-L-glutamate binding 58 times more tightly in the presence of 2,4-diaminopyrimidine than in its absence. In the presence of coenzymes, this cooperativity ranges from 1.8- to 428-fold. The effects of coenzymes on individual binding steps range from an 8-fold decrease in binding constant to a 23-fold increase. The structural specificity of these effects are discussed in terms of a model involving ligand-induced conformational changes and compared with the effects on trimethoprim and methotrexate binding described in the preceding paper [Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980) *Biochemistry* (first paper of four in this issue)].

In investigations of the interactions of relatively complex small molecules with proteins, valuable clues to the origins of the specificity of the interactions can come from studies of molecules which can be regarded as "fragments" of the ligand of interest. Thus, in the course of our studies of the binding of the antifolate drug methotrexate to dihydrofolate reductase [see Roberts et al. (1977) and Roberts (1978)], we examined the binding of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate as fragments of methotrexate. Although 2,4-diaminopyrimidine is not strictly a fragment of methotrexate, there is evidence [see Birdsall et al. (1978)] that these two compounds and the corresponding parts of methotrexate do bind to the enzyme in a similar manner. They were found to bind not only simultaneously but *cooperatively* to the enzyme, in that *p*-aminobenzoyl-L-glutamate binds substantially more tightly in the presence than in the absence of 2,4-diaminopyrimidine (Birdsall et al., 1977, 1978).

This cooperativity between subsites of the inhibitor binding site was not dramatically affected by NADPH, although clear evidence was obtained that the two kinds of cooperativity (between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine, and between these two ligands and NADPH) are not independent (Birdsall et al., 1978). In view of the substantial effects of a series of coenzyme analogues on the binding of methotrexate, reported in the preceding paper (Birdsall et al., 1980), we have now examined the effects of these analogues on the binding of the two methotrexate fragments.

Experimental Section

Materials

The sources of enzyme, coenzymes, and other ligands are given by Birdsall et al. (1978, 1980); abbreviations used for the coenzyme analogues are those of the preceding paper (Birdsall et al., 1980). Binding constants were measured fluorometrically by modifications of the procedures described in detail by Birdsall et al. (1978, 1980). All measurements were made at 25 °C, in a buffer of 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol]-hydrochloride, pH 6.0, containing 0.5 M KCl, by using a Perkin-Elmer MPF-44A fluorescence spectrometer, with 1-cm path length quartz cells.

Methods

The binding of *p*-aminobenzoyl-L-glutamate to the enzyme–coenzyme or enzyme–coenzyme–2,4-diaminopyrimidine complexes was followed by measuring the increase in the fluorescence of *p*-aminobenzoyl-L-glutamate on binding (Birdsall et al., 1978; excitation, 320–340 nm; emission, 350–355 nm) or, for complexes with reduced coenzymes, the change in the coenzyme fluorescence (excitation, 350–380 nm; emission, 400–470 nm). 2,4-Diaminopyrimidine binding was followed by measuring changes in the fluorescence of the enzyme (excitation, 310 nm; emission, 330–345 nm; enzyme–oxidized coenzyme complexes) bound *p*-aminobenzoyl-L-glutamate (excitation, 320–335 nm; emission, 350–355 nm; enzyme–oxidized coenzyme–*p*-aminobenzoyl-L-glutamate complexes) or reduced coenzyme (excitation, 350–380 nm; emission, 400–470 nm) or in the energy transfer fluorescence of the reduced coenzyme (excitation of tryptophan residues at 310 nm; emission from the reduced nicotinamide ring at 400–450 nm). The binding of ϵNADP$^+$ was followed by the increase in its fluorescence (excitation, 330–350 nm; emission, 390–400 nm), and that of other oxidized coenzymes was followed by competition with ϵNADP$^+$.

Corrections for self-absorption were made and binding constants were calculated as described by Birdsall et al. (1978, 1980) by using nonlinear regression.

Thermodynamic Relationships. The equilibria describing the binding of *p*-aminobenzoyl-L-glutamate (P), 2,4-diaminopyrimidine (D), and coenzyme (C) to dihydrofolate reductase are shown in Scheme I. The equilibrium constants are defined as binding constants, so that

$$K_{E,P} = [EP]/[E][P] \quad (1)$$

$$K_{ED,P} = [EDP]/[ED][P] \quad (2)$$

$$K_{ECD,P} = [ECDP]/[ECD][P] \quad (3)$$

and so on, where the notation used is such that $K_{ED,P}$ is the

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Table I: Binding Constants[a] of Oxidized Coenzymes, *p*-Aminobenzoyl-L-glutamate, and 2,4-Diaminopyrimidine to Dihydrofolate Reductase

coenzyme	$K_{E,C}$[b] (M^{-1})	$K_{EP,C}$ (M^{-1})	$K_{ED,C}$ (M^{-1})	$K_{EDP,C}$ (M^{-1})	$K_{EC,P}$ (M^{-1})	$K_{EC,D}$ (M^{-1})	$K_{ECP,D}$ (M^{-1})	$K_{ECD,P}$ (M^{-1})
none[c]					8.1×10^2	1.25×10^3	7.25×10^4	4.7×10^4
$NADP^+$	6.1×10^4	2.5×10^5	1.0×10^6	1.3×10^5	(3.3×10^3)	(2.2×10^4)	(3.8×10^4)	(6.0×10^3)
$NHDP^+$	9.4×10^3	(2.9×10^4)	1.4×10^5	(3.7×10^4)	2.5×10^3	(1.8×10^4)	9.2×10^4	(1.3×10^4)
$\epsilon NADP^+$	4.9×10^4	1.8×10^5	7.1×10^5	1.45×10^5	(3.0×10^3)	1.8×10^4	(5.8×10^4)	(9.6×10^3)
$TNADP^+$	1.4×10^4	(8.9×10^3)	2.7×10^4	(1.3×10^5)	5.2×10^2	2.4×10^3	1.0×10^6	(2.2×10^5)
$APADP^+$	8.9×10^3	(3.5×10^4)	5.0×10^4	(9.7×10^4)	3.25×10^3	(7.0×10^3)	2.0×10^5	(9.2×10^4)
PADPR-OMe	5.2×10^4	(5.1×10^4)	(8.6×10^4)	(8.9×10^4)	8.0×10^2	2.1×10^3	1.3×10^5	(4.9×10^4)

[a] The notation for the equilibrium constants is indicated on Scheme I. Estimated precision is ±20%. Values in parentheses were not determined directly but were calculated by using eq 4–9. The remaining equilibrium constants in Scheme I can be calculated similarly. [b] From Birdsall et al. (1980). [c] From Birdsall et al. (1978).

Table II: Binding Constants[a] of Reduced Coenzymes, *p*-Aminobenzoyl-L-glutamate, and 2,4-Diaminopyrimidine to Dihydrofolate Reductase

coenzyme	$K_{E,C}$[b] (M^{-1})	$K_{EC,P}$ (M^{-1})	$K_{EC,D}$ (M^{-1})	$K_{ECP,D}$ (M^{-1})	$K_{ECD,P}$ (M^{-1})
none[c]		8.1×10^2	1.25×10^3	7.25×10^4	4.7×10^4
NADPH[d]	1.0×10^8	3.8×10^3	1.1×10^4	2.7×10^5	(9.1×10^4)
NHDPH	3.1×10^7	1.4×10^3	8.6×10^3	4.7×10^5	7.6×10^4
ϵNADPH	3.1×10^7	1.4×10^3	1.0×10^4	4.1×10^5	(5.6×10^4)
TNADPH	2.0×10^5	7.0×10^2	1.6×10^4	1.3×10^5	(5.75×10^3)
APADPH	2.1×10^6	2.4×10^3	2.9×10^4	8.8×10^5	(7.2×10^4)

[a] The notation for the equilibrium constants is indicated on Scheme I. Estimated precision is ±20%. Values in parentheses were not determined directly but were calculated by using eq 4–9. The remaining equilibrium constants in Scheme I can be calculated similarly. [b] From Birdsall et al. (1980). [c] From Birdsall et al. (1978). [d] In good agreement with the results of Birdsall et al. (1978).

equilibrium constant for the binding of *p*-aminobenzoyl-L-glutamate (P) to the enzyme–2,4-diaminopyrimidine complex (ED). The same subscripts will be used for the corresponding Gibbs free energy changes, $\Delta G°$.

As indicated in Scheme I, 12 separate equilibria can be distinguished in a system involving the binding of three ligands to a protein. However, these are not, of course, all independent. Simple thermodynamic arguments (Wyman, 1948, 1964; Weber, 1975) show that

$$K_{E,P}K_{EP,D} = K_{E,D}K_{ED,P} \quad (4)$$

$$K_{EC,P}K_{ECP,D} = K_{EC,D}K_{ECD,P} \quad (5)$$

$$K_{E,P}K_{EP,C} = K_{E,C}K_{EC,P} \quad (6)$$

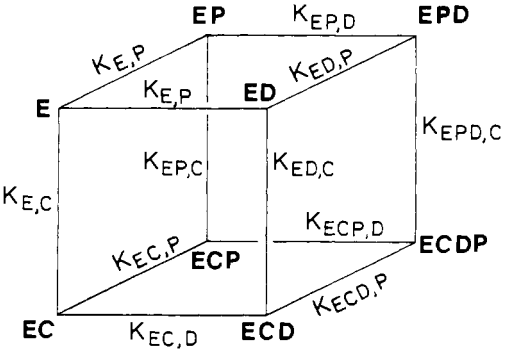
$$K_{E,D}K_{ED,C} = K_{E,C}K_{EC,D} \quad (7)$$

$$K_{EP,D}K_{EPD,C} = K_{EP,C}K_{ECP,D} \quad (8)$$

$$K_{ED,P}K_{EPD,C} = K_{ED,C}K_{ECD,P} \quad (9)$$

Five of these relationships are independent, and it follows that seven independent equilibrium constants suffice to describe the system. Four of these are known from our earlier work. Birdsall et al. (1978) determined the four equilibrium constants $K_{E,D}$, $K_{E,P}$, $K_{ED,P}$, and $K_{EP,D}$ (three of which are independent), and $K_{E,C}$ values for the various coenzyme analogues are given in the preceding paper (Birdsall et al., 1980). To complete the description of the system of Scheme I, it is therefore sufficient to measure *any* three of the remaining eight equilibrium constants. The choice of which three to measure was governed by experimental convenience—largely by the magnitude of the observed fluorescence changes—and varied according to which coenzyme analogue was being studied. The relatively tight binding of the reduced coenzymes made it easier to measure the binding of P and/or D to the enzyme–coenzyme complex rather than vice versa. Since equilibrium constants such as $K_{ECD,P}$ refer to a situation where the enzyme is *wholly* saturated with D and C, a situation which is not experimentally accessible, the measured equilibrium constants were corrected for incomplete saturation with the first (and, where relevant, second) ligands by using equations analogous to eq 10 of Birdsall et al. (1978).

Scheme I: Equilibria Involved in the Binding of Coenzyme (C), 2,4-Diaminopyrimidine (D), and *p*-Aminobenzoyl-L-glutamate (P) to Dihydrofolate Reductase (E), Showing the Nomenclature Used for the Equilibrium Constants



Results and Discussion

The measured binding constants are given in Tables I and II. As described above, only three further equilibrium constants, in addition to those already known (Birdsall et al., 1978, 1980), are required to completely describe the system for each coenzyme analogue. Since experimental considerations dictated that the three equilibrium constants measured were not the same for each coenzyme analogue, we have included in Table I values of all the relevant equilibrium constants (calculated where necessary from eq 4–9) to facilitate comparisons between the different coenzymes. The same three equilibrium constants were measured for all the reduced coenzyme complexes, so that comparisons in Table II can be made directly.

The standard deviations of the measured equilibrium constants obtained from the nonlinear regression analysis were in the range 5–15%. We showed earlier (Birdsall et al., 1978) that in this system the standard deviations estimated from this analysis were comparable to those derived from replicate determinations and thus a reasonable estimate of the precision of the equilibrium constants. However, in the present case all the equilibrium constants incorporate a correction for incomplete saturation with ligand(s) present at fixed concentrations, and this requires a knowledge of at least one additional equilibrium constant. These corrections were in general

Table III: Effects of Coenzymes on the Binding of *p*-Aminobenzoyl-L-glutamate and 2,4-Diaminopyrimidine to Dihydrofolate Reductase

coenzyme	$K_{EC,P}/K_{E,P}$	$K_{EC,D}/K_{E,D}$	$K_{ECP,D}/K_{EP,D}$	$K_{ECD,P}/K_{ED,P}$	K_{coop}[a]
$NADP^+$	4.1	17.2	0.53	0.13	1.8
$NHDP^+$	3.1	14.5	1.3	0.27	5.1
$\epsilon NADP^+$	3.7	14.6	0.80	0.20	3.2
$TNADP^+$	0.64	1.9	14.3	4.7	428
$APADP^+$	4.0	5.6	2.7	1.9	28
PADPR-OMe	1.0	1.6	1.7	1.0	61
NADPH	4.6	8.9	3.7	1.9	24
NHDPH	1.7	6.9	6.5	1.6	54
ϵNADPH	1.7	8.0	5.7	1.2	41
TNADPH	0.86	12.9	1.8	0.12	8.0
APADPH	3.0	23.4	12.1	1.5	30

[a] $K_{coop} = K_{ECD,P}/K_{EC,P} = K_{ECP,D}/K_{EC,D}$. In the absence of coenzyme, $K_{coop} = 58$.

small, but taking them into account we conclude that a reasonable conservative estimate of the precision of the values in Tables I and II is ±20%. For two of the coenzymes, $\epsilon NADP^+$ and NHDPH, we have been able to measure four rather than three equilibrium constants, so that (by means of eq 4–9) an internal check on the precision of the measurements is available. Thus, for NHDPH

$$K_{EC,P}K_{ECP,D} = 6.6 \times 10^8\ M^{-2}$$

$$K_{EC,D}K_{ECD,P} = 6.5 \times 10^8\ M^{-2}$$

indicating precision well within ±20%.

The values in Tables I and II show that the presence of coenzyme analogues does significantly affect the binding of *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine. The effects are not as large as some of those reported in the preceding paper (Birdsall et al., 1980; a maximum effect of a factor of 23 is seen here), but they do depend quite markedly on the structure of the coenzyme analogue, so that, for example, the equilibrium constant for the binding of *p*-aminobenzoyl-L-glutamate to the enzyme–coenzyme–2,4-diaminopyrimidine complex varies over a 36-fold range, depending on the coenzyme involved. It is particularly notable that a number of instances of negative cooperativity are observed; for example, *p*-aminobenzoyl-L-glutamate binds 8 times more weakly to the enzyme–$NADP^+$–2,4-diaminopyrimidine complex than to the enzyme–2,4-diaminopyrimidine complex. (Negative cooperativity and competition were unambiguously distinguished by examining the effect of the concentration of one ligand on the apparent binding constant of the other; Birdsall et al., 1978.)

Effects of Oxidized Coenzymes on Individual Binding Steps. In order to make it easier to compare the effects of the different coenzyme analogues on the binding of the two fragments of methotrexate, we show in Table III the ratios of the four equilibrium constants describing the fragment binding in the presence of coenzyme to the equivalent equilibrium constants in the absence of coenzyme. Table III also gives values of K_{coop}, the factor by which *p*-aminobenzoyl-L-glutamate increases the binding of 2,4-diaminopyrimidine (and vice versa). It is clear that K_{coop} varies very markedly as the nature of the coenzyme is changed, so that not only the binding of the individual fragments but also the interaction between them are affected by the coenzyme.

PADPR-OMe, the coenzyme analogue in which the nicotinamide ring has been replaced by a methoxy group, has the least effect of any of the analogues examined. It has no effect on the binding of *p*-aminobenzoyl-L-glutamate, either in the presence or in the absence of 2,4-diaminopyrimidine, has only a small effect on the binding of 2,4-diaminopyrimidine, and leaves the cooperativity between them entirely unaffected. This suggests that the substantially larger effects of the other coenzyme analogues must be primarily associated with the nicotinamide ring. This is clearly consistent with the results of the preceding paper (Birdsall et al., 1980), and, in the enzyme–NADPH–methotrexate complex, the nicotinamide ring is the only part of the coenzyme which is close to the inhibitor (Matthews et al., 1979).

Further evidence for the nicotinamide ring as the major determinant of these influences on fragment binding comes from a comparison of the effects of $NADP^+$, $NHDP^+$, and $\epsilon NADP^+$. These three coenzymes, which differ at the adenine but not at the nicotinamide end, have closely similar effects on the four individual steps of fragment binding (Table III). The binding of *p*-aminobenzoyl-L-glutamate is increased 3-4-fold and that of 2,4-diaminopyrimidine is increased 14-17-fold. These figures apply to the simple binary complex; it is striking that when one of the fragments is already bound, $NADP^+$ and related coenzymes have much less favorable effects on the binding of the second fragment. When 2,4-diaminopyrimidine is the second fragment, $NADP^+$ *decreases* its binding constant by almost a factor of 2, while with *p*-aminobenzoyl-L-glutamate in the same situation the decrease is eightfold. Coenzymes bearing a normal oxidized nicotinamide ring thus increase the binding of both fragments to the enzyme alone, but when another fragment is already bound, they tend to have the opposite effect. As a corollary, the cooperativity between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine is greatly decreased by these coenzymes, from 58 to between 2 and 5.

As we observed for methotrexate and trimethoprim binding (Birdsall et al., 1980), modification of the nicotinamide ring substantially alters the effects of the coenzyme on inhibitor binding. In the present instance, replacement of the carboxamide by a thioamide to give $TNADP^+$ leads to an interesting "inversion" of the coenzyme effects. $TNADP^+$ has rather little effect on the binding of either *p*-aminobenzoyl-L-glutamate or 2,4-diaminopyrimidine to the enzyme alone, but it does significantly increase the binding of either of them when the other is already present (compare $K_{ECP,D}/K_{EP,D} = 14.3$ and $K_{EC,D}/K_{E,D} = 1.9$). The cooperativity between the two fragments is thus substantially increased, to the striking value of 428 (equivalent to 3.6 kcal/mol).

The acetylpyridine analogue, $APADP^+$, shows behavior intermediate between that of $NADP^+$ and that of $TNADP^+$. It produces a modest increase in the binding constant of both fragments, both to the enzyme alone and in the presence of the other fragment. Since the effects are similar for each binding step, the cooperativity between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine is not greatly affected (a factor of 2).

The clear distinction between TNADP$^+$ and APADP$^+$ on the one hand and NADP$^+$, NHDP$^+$, and ϵNADP$^+$ on the other has been discussed in the preceding paper (Birdsall et al., 1980) and is also apparent in the ^{1}H and ^{31}P chemical shifts of the bound coenzymes in their binary complexes with the enzyme (Hyde et al., 1980a,b). In particular, the changes in chemical shifts of the nicotinamide ring protons of APADP$^+$ on binding are intermediate between those of TNADP$^+$ and those of NADP$^+$ itself (Hyde et al., 1980a). Here again, as noted in the preceding paper (Birdsall et al., 1980) for the ternary complexes with methotrexate, folate and trimethoprim, there is a correlation, albeit as yet a purely qualitative one, between these chemical shift changes and the binding characteristics of the various coenzyme analogues. This correlation indicates that it is the different mode of binding of the modified nicotinamide ring in TNADP$^+$ and APADP$^+$ (compared to NADP$^+$) demonstrated by the NMR experiments which is responsible for their different effects on the binding of the fragments of methotrexate.

Effects of Reduced Coenzymes on Individual Binding Steps. In the preceding paper (Birdsall et al., 1980), we showed that reduced coenzymes have a much larger effect on the binding of methotrexate and trimethoprim than do their oxidized counterparts. This is not the case for the binding of *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine; the order of magnitude of the effects of reduced and oxidized coenzymes is very similar. However, the specificity of their effects on the individual binding steps is quite distinct.

The three coenzymes with normal nicotinamide rings, NADPH, NHDPH, and ϵNADPH, produce modest increases in the binding constants of both *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine, in the presence or the absence of the second fragment. The effect on 2,4-diaminopyrimidine binding is generally greater than on *p*-aminobenzoyl-L-glutamate binding (although NADPH has a rather larger effect on the binding of *p*-aminobenzoyl-L-glutamate to the enzyme alone than do NHDPH and ϵNADPH). This is particularly noticeable with APADPH; like NADPH, this coenzyme analogue leads to a small (1.5–3-fold) increase in *p*-aminobenzoyl-L-glutamate binding, but it produces much larger effects (12–23-fold) on the binding of 2,4-diaminopyrimidine. The latter effects are 2.5–3 times larger than those produced by NADPH, and the 23-fold increase in the binding of diaminopyrimidine to the enzyme alone is the largest effect of any of the coenzymes on the binding of a methotrexate fragment (equivalent to 1.9 kcal/mol). It is also striking that for this group of reduced coenzymes the effects on fragment binding are not greatly influenced by the presence of the second fragment, whereas the effects of the corresponding oxidized coenzymes, NADP$^+$, NHDP$^+$, and ϵNADP$^+$, are often reversed in sign by the presence of the second fragment.

Among the reduced, as among the oxidized, coenzymes, the thionicotinamide analogue stands out alone. Here the effects of the coenzyme *do* depend on the presence or absence of the second fragment. Addition of *p*-aminobenzoyl-L-glutamate converts a large increase in 2,4-diaminopyrimidine binding into a small one. Similarly, with the enzyme alone, TNADPH has little if any effect on *p*-aminobenzoyl-L-glutamate binding, but in the presence of 2,4-diaminopyrimidine it leads to a pronounced *decrease* in binding. Because TNADPH produces either a smaller increase or a decrease in binding in the ternary complexes, it leads to a marked decrease in the cooperativity between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine, from 58 to 8. This is in contrast to the behavior of the other reduced coenzymes, which decrease K_{coop} by no more than about a factor of 2. In fact, in terms of its effects on the binding of the methotrexate fragments, TNADPH behaves more like NADP$^+$ than like NADPH.

Table IV: Overall Effects of Coenzyme Analogues on the Binding of *p*-Aminobenzoyl-L-glutamate and 2,4-Diaminopyrimidine to Dihydrofolate Reductase

coenzyme	$\Delta\Delta G°$ [a] (kcal/mol)	coenzyme	$\Delta\Delta G°$ [a] (kcal/mol)
NADP$^+$	−0.4	NADPH	−1.6
NHDP$^+$	−0.8	NHDPH	−1.4
ϵNADP$^+$	−0.6	ϵNADPH	−1.3
TNADP$^+$	−1.3	TNADPH	−0.2
APADP$^+$	−1.4	APADPH	−1.9
PADPR-OMe	−0.3		

[a] $\Delta\Delta G° = \Delta G°_{EC,DP} - \Delta G°_{E,DP}$; see eq 10–12.

Overall Effects of Coenzymes on Fragment Binding. The overall change in Gibbs free energy for the process

$$E + D + P \rightleftharpoons EDP \qquad (10)$$

(where E, D, and P represent enzyme, 2,4-diaminopyrimidine, and *p*-aminobenzoyl-L-glutamate, respectively) is given by the sum of the changes in the two component binding steps:

$$\Delta G°_{E,DP} = \Delta G°_{E,D} + \Delta G°_{ED,P} = \Delta G°_{E,P} + \Delta G°_{EP,D} \qquad (11)$$

The subscripts have the same meaning as for the corresponding equilibrium constants in Scheme I, and the second equality in eq 11 is equivalent to that in eq 4. The change in this parameter produced by the coenzymes

$$\Delta\Delta G° = \Delta G°_{EC,DP} - \Delta G°_{E,DP}$$

is thus a measure of their overall effect on the binding of the two fragments of methotrexate; values of $\Delta\Delta G°$ are listed in Table IV. From the data of Birdsall et al. (1978) reproduced in Table I, $\Delta G°_{E,DP} = -10.6$ kcal/mol; from Table IV, the mean value of $\Delta\Delta G°$ is −1.02 kcal/mol, so that the overall effect of the coenzymes on fragment binding is relatively small.

It can be seen from Table IV that the overall effect of the various coenzymes is also relatively uniform: $\Delta\Delta G°$ has a standard deviation of ±0.58 kcal/mol and a range of −0.2 to −1.9 kcal/mol. This contrasts with the changes produced by the coenzymes in $\Delta G°$ for the individual binding steps, which range from +1.25 to −1.86 kcal/mol. The variations in the cooperativity between *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine are particularly marked; K_{coop} varies from 1.8 to 428 ($\Delta G°_{coop}$ varies from −0.35 to −3.58 kcal/mol). As an example, compare PADPR-OMe with NADP$^+$ or TNADPH. All three have very similar and very small overall effects ($\Delta\Delta G°$ = −0.2 to −0.4 kcal/mol). In the case of PADPR-OMe, the effects on individual steps are also small (≤0.3 kcal/mol), but with NADP$^+$ and TNADPH the small overall effects arise from the near cancellation of much larger (up to 1.7 kcal/mol) positive and negative effects on the component steps.

The interpretation of these effects will obviously depend on the kind of mechanism postulated to account for the cooperativity in ligand binding in this system. In discussing the cooperativity between 2,4-diaminopyrimidine and *N*-(*p*-alkylaminobenzoyl)-L-glutamate (Birdsall et al., 1978), we noted that the observation of *negative* cooperativity is more readily accommodated by a model based on ligand-induced conformational change than by one involving solely ligand–ligand interactions in the ternary complex. This clearly also applies to the effects of coenzymes discussed here; in the preceding paper (Birdsall et al., 1980) we have summarized the evidence suggesting that the coenzyme effects on methotrexate and

trimethoprim binding are conformational in origin. Within the framework of a model based on conformational change, there are two possible interpretations of the relative constancy of $\Delta\Delta G°$ values for the different coenzymes. The first is that it is essentially coincidental: the various coenzymes produce different conformational changes, so that the conformation of the enzyme–coenzyme–2,4-diaminopyrimidine–*p*-aminobenzoyl-L-glutamate quaternary complex is different in each case, but the consequences in terms of binding energy are accidentally similar in each case. Particularly in view of the fact that the values of $\Delta\Delta G°$ are small, this possibility certainly cannot be ruled out at present. An alternative, however, is that the similar values of $\Delta\Delta G°$ do in fact imply similar structures for the quaternary complexes. The marked variation in Gibbs energy changes for the individual steps would then imply that the final structure of the quaternary complex could be arrived at in a number of different ways, depending on the Gibbs energy balance in each of the binary and ternary complexes. In order to distinguish between these two possibilities, structural comparisons of the various quaternary complexes (for example, by NMR spectroscopy) will obviously be required. For the enzyme–oxidized coenzyme–methotrexate complexes, it seems very likely that the conformation is *not* the same for all the coenzyme analogues (Hyde et al., 1980b). However, the differences in the behavior of methotrexate and its fragments in the presence of coenzyme (see below) are such that this is probably not a reliable guide to the characteristics of the quaternary complexes.

Mechanism of Cooperativity. We have earlier proposed a simple model to explain the cooperativity in binding observed between 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate (Birdsall et al., 1978). In this model, the enzyme is supposed to exist in two conformational states, E_A and E_B; E_A predominates in the absence of ligands, while 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate both bind more tightly to E_B. The binding of the first ligand thus increases the proportion of the enzyme in the E_B conformation, and the second ligand binds more tightly. Some support for this simple model has come from ^1H NMR studies of the binding of *N*-(*p*-alkylaminobenzoyl)-L-glutamates (Birdsall, 1978). A detailed comparison of the ^1H spectra of a homologous series of ternary complexes (enzyme–2,4-diaminopyrimidine–*N*-(*p*-alkylaminobenzoyl)-L-glutamate) indicated that only two conformational states had to be postulated, one favored by the longer chain *N*-alkyl compounds (which show negative cooperativity) and the other favored by *p*-aminobenzoyl-L-glutamate itself. The amplitude of the difference spectra, which would be an estimate of the proportions of the two conformations, correlated well with the degree of cooperativity (Birdsall, 1978).

The simplest way in which the binding of the fragments could be influenced by coenzyme would then be for coenzyme binding to alter the proportions of E_A and E_B. (This of course corresponds to the model in which all the quaternary complexes have the essentially same conformation, namely E_B.) An increase in the relative amount of E_B would increase the binding constants of both fragments and decrease the cooperativity between them. However, this could not explain the observation that several coenzymes (e.g., $NADP^+$) produce increases in some binding constants and decreases in others. In fact, analysis of the data in Tables I and II in terms of this model, using the equations given by Birdsall et al. (1978), shows clearly that for none of the coenzyme analogues can the effects on fragment binding be explained by postulating that the only effect of coenzyme binding is to change the equilibrium constant for the interconversion of E_A and E_B.

There must, in addition, be specific effects of the coenzymes on the binding of diaminopyrimidine and/or *p*-aminobenzoyl-L-glutamate to E_A and/or E_B. No detailed description of these effects is possible, however, since even in this simple model the number of equilibria substantially exceeds the number of independently measurable binding constants. A large number of combinations of equilibrium constants can therefore be found to fit the data: indeed, the data only define either upper or lower limits to the individual equilibrium constants. Independent estimates of some of the parameters in the model—for example, of the relative proportions of the E_A and E_B conformations—will be required before it can be described in sufficient detail to be testable.

Comparison with Trimethoprim and Methotrexate Binding. It is impossible to make a quantitative comparison between the binding of methotrexate and that of the fragments, because of the difficulties in defining an appropriate standard state and in accounting for the different changes in entropy in the two situations. However, a qualitative comparison of the effects of the coenzymes on the overall binding of the fragments to those on methotrexate binding (Birdsall et al., 1980) is of some interest. The oxidized coenzymes have a similar overall effect in both cases, increasing inhibitor binding by 2–11-fold for the fragments and by 5–13-fold for methotrexate. This is thus generally consistent with the idea that the fragments bind in the same way as the corresponding parts of methotrexate, for which there is a certain amount of spectroscopic evidence in the absence of coenzyme (Birdsall et al., 1977; Feeney et al., 1977; Kimber et al., 1977; Roberts et al., 1977). However, the structural specificity of these effects is not the same; $TNADP^+$ has a larger overall effect than $NADP^+$ on fragment binding, whereas the reverse is true for methotrexate binding. There must, therefore, be at least some differences between the fragment complexes and those of methotrexate. A much more striking difference is seen with the reduced coenzymes. These increase the binding of methotrexate by several hundred-fold (Birdsall et al., 1980), but their overall effect on the binding of the methotrexate fragments is no more than a factor of 40. In the methotrexate–NADPH complex (Matthews et al., 1978), the nicotinamide ring of the coenzyme is in contact with the pyrazine portion of the pteridine ring of methotrexate. It is just this part of methotrexate which is not represented in the fragment complex, so a substantial discrepancy is perhaps not surprising. As noted in the preceding paper (Birdsall et al., 1980), it is unlikely that this nicotinamide–methotrexate contact makes more than a minor contribution to the very much tighter binding in the ternary complex, and the implication is that the similarity between fragment binding and methotrexate binding is much less in the presence than in the absence of reduced coenzyme.

The comparison between the effects of coenzyme on trimethoprim binding and on 2,4-diaminopyrimidine binding is much more straightforward (Table V). Once again, with the oxidized coenzymes, the effects are of the same order of magnitude but differ in structural specificity. Coenzymes having an unmodified nicotinamide ring increase the binding of 2,4-diaminopyrimidine 14–17-fold and that of trimethoprim 2–3-fold, whereas $TNADP^+$, $APADP^+$, and PADPR-OMe have similar or rather greater effects on trimethoprim binding than on that of 2,4-diaminopyrimidine. There is evidence from ^1H and ^{31}P NMR (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) for the coexistence of two conformational states of the enzyme–coenzyme–trimethoprim complexes, the relative

Table V: Comparison of the Effects of Coenzymes on the Binding of Trimethoprim and of 2,4-Diaminopyrimidine to Dihydrofolate Reductase

coenzyme	$K_{EC,D}/K_{E,D}$[a]	$K_{EC,TMP}/K_{E,TMP}$[b]
$NADP^+$	17.2	2.0
$NHDP^+$	14.5	2.9
$\epsilon NADP^+$	14.6	2.5
$TNADP^+$	1.9	6.1
$APADP^+$	5.6	4.8
PADPR-OMe	1.6	4.7
NADPH	8.9	135
NHDPH	6.9	
ϵNADPH	8.0	
TNADPH	12.9	870
APADPH	23.4	195

[a] From Table III. [b] From Birdsall et al. (1980).

populations of the two states being quite different for $NADP^+$ and $TNADP^+$. In contrast, no evidence for two conformational states has been found for 2,4-diaminopyrimidine complexes (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments), so that there is clear evidence for a difference in conformation between the trimethoprim and diaminopyrimidine complexes. On the other hand, the diaminopyrimidine ring of trimethoprim appears to bind in at least a very similar way to 2,4-diaminopyrimidine itself (Cayley et al., 1979). The effects of coenzymes on trimethoprim binding can thus be separated, at least formally, into two distinct processes. The binding of oxidized coenzymes having a normal nicotinamide ring produces a conformational change which leads to ~15-fold tighter binding of the diaminopyrimidine ring. These coenzymes with a missing or modified nicotinamide ring produce a different conformational change(s) (Birdsall et al., 1980; Hyde et al., 1980a,b) which has(have) an appreciably smaller effect. Superimposed on this is the effect of the trimethoxybenzyl substituent. In the case of $NADP^+$, this has an unfavorable effect of about a factor of 8, which is associated with a significant change in coenzyme conformation (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments).

For trimethoprim, as for methotrexate, reduced coenzymes have a very much larger effect on the binding constant than do their oxidized counterparts (Birdsall et al., 1980). Since this effect is not seen with 2,4-diaminopyrimidine, it must originate from the trimethoxybenzyl group. In methotrexate, it appears to be related to the presence of the pyrazine ring (see above), and it is interesting that, in the conformation we have proposed for trimethoprim bound to the enzyme (Cayley et al., 1979), the trimethoxybenzyl ring occupies, very roughly, a similar region of space to that occupied by the pyrazine ring of methotrexate. This is perhaps a further indication that reduced coenzymes produce a conformational change in this part of the substrate binding site. Comparison of the crystal structures of the methotrexate complex of *Escherichia coli* dihydrofolate reductase (Matthews et al., 1977) and the methotrexate–NADPH ternary complex of the *L. casei* enzyme (Matthews et al., 1978) suggests that the conformation of residues 12–21 is altered by NADPH binding so as to bring the side chain of Leu-19 into contact with both the pyrazine portion of the bound methotrexate and the nicotinamide ring of NADPH (Matthews et al., 1978). This conformational change thus seems very likely to contribute to the substantial effect of NADPH on methotrexate binding (Birdsall et al., 1980) and most probably also to that on trimethoprim binding if Leu-19 makes a favorable contact with the trimethoxybenzyl ring.

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