Dihydrofolate Reductase from the L1210R Murine Lymphoma. Fluorometric Measurements of the Interaction of the Enzyme with Coenzymes, Substrates, and Inhibitors*

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ABSTRACT: The quenching of protein fluorescence that occurs when dihydrofolate reductase is titrated with coenzymes (NADPH or $NADP^{+}$, reduced or oxidized nicotinamide–adenine dinucleotide phosphate, respectively) and substrates (folate or dihydrofolate) has been utilized to determine the stoichiometry and dissociation constants of the various complexes. The enzyme binds these reactants in a molar ratio of 1:1 characterized by dissociation constants ranging from 5×10^{-8} M to 9×10^{-7} M. An enzyme–NADPH complex can also be demonstrated from the effect of the enzyme on the fluorescence spectrum of NADPH; the emission maximum is shifted from 465 to 445 mμ with concomitant enhancement of fluorescence intensity. The folate analogs methotrexate and triampterene also quench the fluorescence of the enzyme. The binary complex of enzyme and triampterene is characterized by a dissociation constant of 3.4×10^{-7} M while the dissociation of methotrexate was characterized by a constant with a value less than 10^{-8} M. A ternary complex between NADPH, enzyme, and either inhibitor was observed. When NADPH and triampterene form a ternary complex, mutual stabilization of binding occurs that results in a 25–60-fold increase in the affinity of the enzyme for both the coenzyme and inhibitor over the affinity measured in binary complexes.

Several pyridine nucleotide coenzyme-linked dehydrogenases have been shown to form complexes with both the oxidized and reduced forms of the coenzymes, as well as with coenzyme analogs. These interactions, which take place in the absence of the other substrate molecule, result in alterations of the fluorescence spectrum of the enzyme and also of the fluorescence spectrum of the reduced coenzyme (Boyer and Theorell, 1956; Velick, 1958; Winer and Schwert, 1958; Theorell and Langan, 1960; Langan, 1960; and McKay and Kaplan, 1964). These changes in fluorescence have been used to determine the stoichiometry and dissociation constants of the various complexes. The agreement between dissociation constants determined in this direct manner and those determined from kinetic measurements have strengthened the concept that the observed interactions are kinetically significant (Theorell and McKinley-McKee, 1961).

Dihydrofolate (FH_2)[1] reductase[2] is a pyridine nucleotide coenzyme-linked dehydrogenase; it catalyzes reaction 1 in a reversible manner although the equilibrium strongly favors the formation of FH_4 (Mathews and Huennekens, 1963).

$$FH_2 + NADPH + H^+ \rightleftharpoons FH_4 + NADP^+ \quad (1)$$

The availability of enzyme preparations of high purity has allowed an investigation of the interaction of this enzyme with its coenzyme forms, substrates, and inhibitors by virtue of alterations of the fluorescence spectra of the enzyme, NADPH (reduced nicotinamide–adenine dinucleotide phosphate), and the inhibitor triampterene. These measurements have made it possible to determine the stoichiometry and dissociation constants of the various complexes.

According to the kinetic mechanism thought to characterize most pyridine nucleotide dehydrogenases, the noncoenzyme substrate does not bind to the free enzyme but only to the previously formed enzyme–coenzyme complex (see Bloomfield *et al.*, 1962). Thus, the observation of a direct interaction between free FH_2 reductase and the substrate FH_2, reported below, is of particular interest with respect to the kinetic mechanism of this enzyme. This report also presents evidence to indicate that ternary complexes are formed between enzyme, coenzyme, and inhibitors.

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[1] Abbreviations used in this work: FH_2, dihydrofolate; FH_4, tetrahydrofolate; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphate, respectively.

[2] 5,6,7,8-Tetrahydrofolate:NAD^+ ($NADP^+$) oxidoreductase E.C.1.5.1.3.

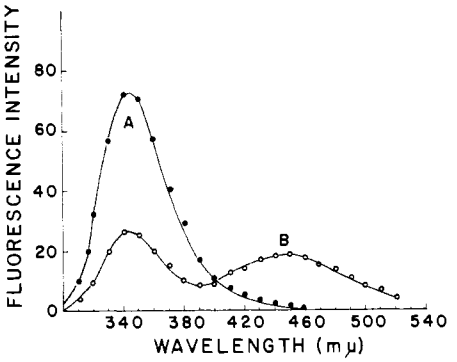

FIGURE 1: Emission spectrum of FH_2 reductase and the enzyme–NADPH complex activated at 290 mμ. Curve A presents the spectrum of free enzyme (2.8×10^{-7} M), while curve B shows the spectrum of the same concentration of enzyme in the presence of 3.6×10^{-7} M NADPH. The buffer was 0.045 M Tris–HCl, pH 7.5, containing 0.068 M KCl. The total volume was 1.1 ml. The meter multiplier was set at 0.03. Under these conditions, 3.6×10^{-7} M NADPH alone did not fluoresce detectably.

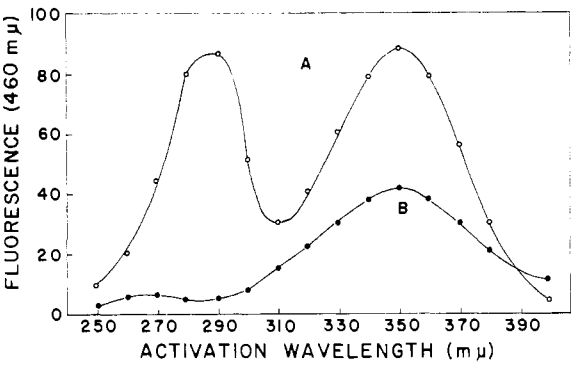

FIGURE 2: Activation spectrum of NADPH and the enzyme–NADPH complex. The emission intensity was measured at 460 mμ. Curve A describes the spectrum of 9×10^{-7} M NADPH in the presence of 5.6×10^{-7} M FH_2 reductase, while Curve B depicts the spectrum of NADPH, 9×10^{-7} M, alone. The buffer was the same as in Figure 1. The meter multiplier was set at 0.01.

Experimental Section

Materials. The studies reported here were performed with FH_2 reductase of 90% purity. Enzyme concentration was determined by titration of enzyme activity with methotrexate (Werkheiser, 1961). Calculation of enzyme purity was based on a molecular weight of 20,000[3] (see Bertino *et al.*, 1965), and protein determinations according to the method of Waddell (1956). Cells of a methotrexate-resistant line of a murine lymphoma, L1210, were used as the source of the enzyme. The purification procedure employed and the properties of this enzyme have been reported in a preliminary communication (Perkins and Bertino, 1965) and will be described in detail elsewhere.[3]

Folic acid was purchased from Nutritional Biochemical Co.; $NADP^+$ (oxidized nicotinamide–adenine dinucleotide phosphate) and NADPH were obtained from Sigma Chemical Co. Methotrexate (amethopterin; 4-amino-10-methylpteroylglutamate), a gift of the Lederle Laboratories, courtesy of Dr. J. Ruegssegger, was purified on DEAE–cellulose (Johns *et al.*, 1961). Chromatographically pure triampterene (2,4,7-triamino-6-phenylpteridine) was a gift of Dr. A. Maass of the Smith Kline and French Laboratories. Dihydrofolate was prepared from folic acid by dithionite reduction (Friedkin *et al.*, 1962).

Methods. All fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer. The instrument was equipped with a 150-w Hanovia Mercury-Xenon light source and an RCA 1P21 photomultiplier tube. The sensitivity setting was kept at 50 and the meter multiplier was varied as indicated in the legends of the figures. Slit arrangement No. 3 was used throughout these experiments. Quartz cuvets with a 1-cm light path containing approximately 1 ml of solution were used for fluorescence measurements. All measurements were made at room temperature (23–25°) and samples were removed from the cell compartment after each reading to prevent heating of the solution by the light source. All wavelengths reported are uncorrected. Titration experiments were carried out by adding small volumes (0.001–0.05 ml) of the titrant delivered by the use of microsyringes (Hamilton Co., Inc.). Corrections were applied when changes in volume upon addition of the titrant were significant.

Results

Fluorescence Emission Spectrum of FH_2 Reductase and the Enzyme–NADPH Complex. The protein emission spectrum for FH_2 reductase is shown in Figure 1 (curve A). The excitation was at 290 mμ and the emission maximum was 345–350 mμ typical of the fluorescence spectra reported for other proteins (Udenfriend, 1962). The effect of NADPH on the enzyme emission spectrum also is illustrated in Figure 1 (curve B). The nucleotide quenched the fluorescence from 300–400 mμ with no shift of the maximum.

Curve B, with a new maximum at 445 mμ, also illustrates the phenomenon of activation by energy transfer previously described by Velick (1958) for lactate and glyceraldehyde 3-phosphate dehydrogenases. Under the conditions employed, the same concentration of free NADPH produced no measurable fluorescence at 445 mμ. Thus, the new fluorescence peak, with a maximum at 445 mμ, is a property of the complex formed between enzyme and NADPH. This effect is illustrated also in Figure 2, which shows the activation spectrum of free NADPH and NADPH in

[3] Manuscript in preparation.

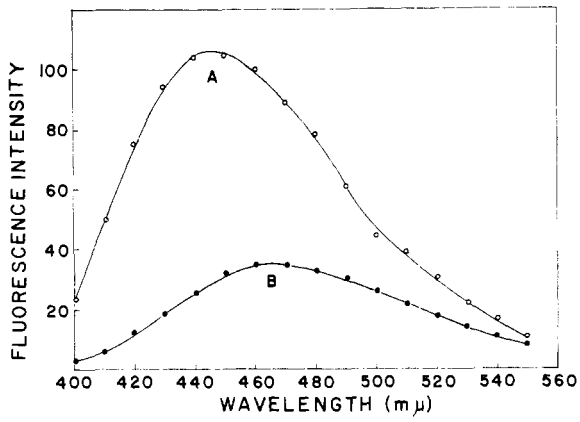

FIGURE 3: Emission spectrum of NADPH and the enzyme–NADPH complex activated at 350 mμ. Curve A shows the spectrum of 9×10^{-7} M NADPH in the presence of 5.6×10^{-7} M FH_2 reductase, while Curve B presents the spectrum of NADPH, 9×10^{-7} M, alone. The buffer was the same as in Figure 1. The meter multiplier was set at 0.01.

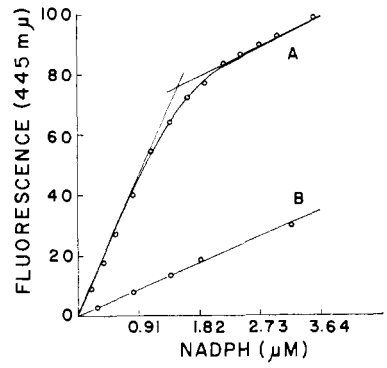

FIGURE 4: Titration of FH_2 reductase with NADPH. Activation was at 350 mμ and emission intensity was measured at 445 mμ. Curve B shows the increase in fluorescence caused by NADPH alone, while Curve A demonstrates the increase in fluorescence observed in the presence of 14×10^{-7} M FH_2 reductase. The equivalence point is indicated by the tangent lines. The buffer was the same as in Figure 1. The meter multiplier was set at 0.03.

the presence of FH_2 reductase as measured at 460 mμ. The activation peaks of free NADPH at 270 and 350 mμ (curve B) correspond to its absorption maxima at 260 and 340 mμ. The new activation maximum at 290 mμ (curve A) corresponds to the activation maximum of free enzyme. As may be noted from Figure 1, however, the fluorescence of free enzyme activated at 290 mμ and measured at 460 mμ is negligible; thus, the new activation peak is a property of the complex.

Fluorescence Emission Spectrum of Free and Bound NADPH. In Figure 3 is illustrated the emission spectrum of free NADPH (curve B) and of the same amounts of NADPH in the presence of FH_2 reductase (curve A). The excitation was at 350 mμ. The enzyme enhanced the fluorescence and shifted the maximum from 465 to 445 mμ. The new emission maximum corresponds to that already demonstrated for the complex in Figure 1.

Stoichiometry and Dissociation Constant for the Enzyme–NADPH Complex. An estimate of the stoichiometry of binding of NADPH to the enzyme was obtained from titration experiments in which small amounts of NADPH were added to a fixed concentration of enzyme. As shown in Figure 4, the fluorescence at 445 mμ is a linear function of NADPH concentration. In the presence of the enzyme, however, the initial slope is markedly increased. Under the conditions of the experiment, *i.e.*, relatively high enzyme concentration, dissociation was slight and a clearly defined inflection occurred that indicated 1 mole of NADPH was bound to 1 mole of enzyme.

Winer *et al.* (1959) have calculated the dissociation constant of the lactate dehydrogenase–NADH complex from the results of such titration experiments. Similar calculations applied to the data from Figure 4 gave a dissociation constant for the FH_2 reductase–NADPH complex of 4×10^{-8} M.

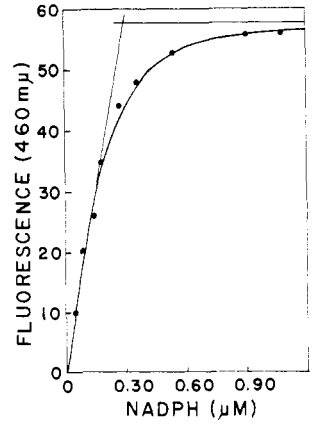

FIGURE 5: Titration of FH_2 reductase with NADPH. Activation was at 290 mμ and emission intensity was measured at 460 mμ. The enzyme concentration was 2.8×10^{-7} M. The curve illustrates NADPH fluorescence activated by energy transfer from the enzyme. The equivalence point is indicated by the tangent lines. The buffer was the same as in Figure 1. The meter multiplier was set at 0.01.

The phenomenon of activation by energy transfer also has been used to determine stoichiometry, as well as the dissociation constant for the enzyme–NADPH complex (Figure 5). Since neither free enzyme nor free NADPH fluoresce significantly under the conditions employed, the observed fluorescence is a direct measure of complex concentration. The experimental points are compared with a theoretical curve plotted from values of (A°) calculated from eq 2 for various values of F when $K_D = 3 \times 10^{-8}$ M, where (E°) is the total enzyme

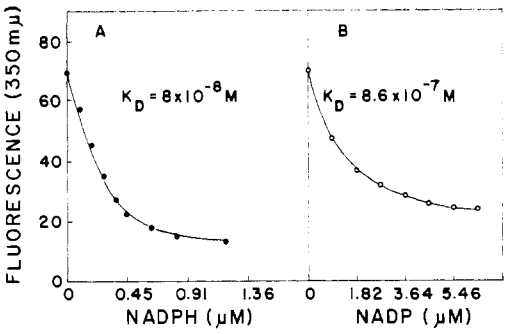

FIGURE 6: Titration of FH_2 reductase with NADPH and NADP. Activation was at 290 mμ and the emission intensity was measured at 350 mμ. The concentration of FH_2 reductase was 2.8×10^{-7} M. Curve A shows the effect of NADPH, and Curve B the effect of $NADP^+$ on the fluorescence of the enzyme. The curves were plotted as described in the text. The buffer was the same as in Figure 1. The meter multiplier was set at 0.03.

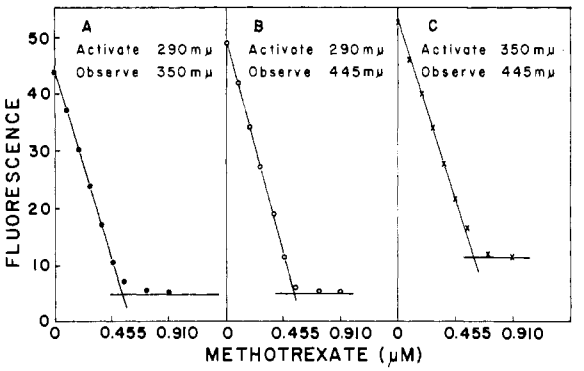

FIGURE 8: Titration of FH_2 reductase and the enzyme–NADPH complex with methotrexate. Activation and emission wavelengths were as indicated in the figures. The concentration of NADPH was 1.82×10^{-6} M under the conditions depicted by curves B and C. The tangent lines indicate the equivalence points. In each case the buffer was the same as in Figure 1. The meter multiplier was set at 0.01 in A and at 0.03 in B and C.

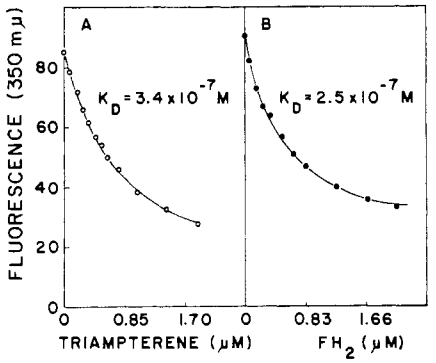

FIGURE 7: Titration of FH_2 reductase with FH_2 and triampterene. Activation was at 290 mμ and the emission intensity was measured at 350 mμ. FH_2 reductase concentration was 2.8×10^{-7} M. Curves A and B indicate the effect of triampterene and FH_2, respectively. The curves were plotted as described in the text. The buffer was the same as in Figure 1. The meter multiplier was set at 0.03

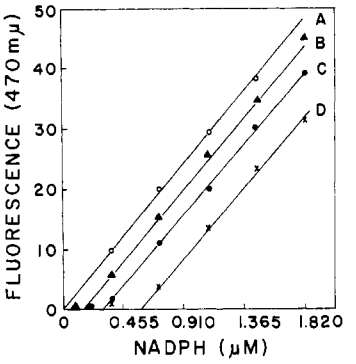

FIGURE 9: Titration of FH_2 reductase–methotrexate complex with NADPH. Activation was at 350 mμ and the emission intensity was measured at 470 mμ. The methotrexate concentration was 9×10^{-7} M in the experiments depicted by curves A, B, C, and D. The enzyme concentration was 1.4×10^{-7} M for curve B, 2.8×10^{-7} M for curve C, and 5.6×10^{-7} M for curve D, while for curve A enzyme was omitted. The buffer was the same as in Figure 1. The meter multiplier was set at 0.01.

concentration, (A°) is the concentration of NADPH added, F_M is the maximum fluorescence obtained, and F is the observed fluorescence.

$$(A^\circ) = K_D/(F_M/F - 1) + (E^\circ)F/F_M \qquad (2)$$

The intersection of the tangent lines indicate a stoichiometry of 1:1.

Effect of NADPH and NADP on Enzyme Fluorescence. When increasing amounts of either NADPH or $NADP^+$ were added to a solution of FH_2 reductase, a quenching of enzyme fluorescence was observed (Figure 6). As was indicated in Figure 1, the degree of quenching was the same throughout the emission spectrum and no shift of the maximum from 350 mμ was observed. In no case was the enzyme fluorescence completely quenched upon addition of the coenzyme forms.

The results illustrated in Figure 6 were used to estimate the respective dissociation constants for the coenzyme forms. The solid lines are theoretical curves

TABLE I: Summary of the Dissociation Constants of the Various Enzyme Complexes at pH 7.5.

Dissociation	K_D (M)	Kinetic Constant	(M)
E[a]–NADPH = E + NADPH	5×10^{-8} [b]	K_m NADPH	5×10^{-6}
E–NADP$^+$ = E + NADP$^+$	9×10^{-7}		
E–FH_2 = E + FH_2	2×10^{-7}	K_m FH_2	4×10^{-7}
E–FA = E + FA	3×10^{-7}		
E–TAM = E + TAM	3×10^{-7}	K_i FA[c]	2×10^{-7}
TAM[a]–E–NADPH = E–NADPH + TAM	5×10^{-9}	K_i TAM	1×10^{-8}
TAM–E–NADP$^+$ = E–NADP$^+$ + TAM	3×10^{-8}		
TAM–E–NADPH = E–TAM + NADPH	2×10^{-9}	K_i NADPH	5×10^{-6}

[a] TAM, triampterene; E, FH_2 reductase. [b] Average of the three values determined from the data in Figures 4–6. [c] FA, folic acid.

plotted from values of (A°) calculated from eq 3[4] for various values of F for K_D as indicated in Figure 6. (A°), (E°), and F have the same significance as in eq 2; $F°$ is the initial fluorescence.

$$(A°) = K_D(F°/F - 1) + (E°)(1 - F/F°) \quad (3)$$

Effect of Substrates and Substrate Analogs on Enzyme Fluorescence. FH_2, the substrate for the purified enzyme, as well as triampterene, a potent inhibitor of this enzyme (Bertino *et al.*, 1965), quenched the fluorescence of the enzyme at low concentrations (Figure 7). The significance of this observation with respect to the kinetic mechanism of FH_2 reductase will be discussed in a later section. Based on the assumption that 1 mole of FH_2 or triampterene binds to 1 mole of enzyme, dissociation constants for the binary complexes were determined as described for the curves in Figure 6. The dissociation constant for folic acid was determined in the same manner (not illustrated). These results are summarized in Table I and indicate that the free enzyme binds all three compounds with similar affinity.

Effect of Methotrexate on Enzyme and Enzyme–NADPH Complex Fluorescence. When methotrexate, a stoichiometric inhibitor of FH_2 reductase (Werkheiser, 1961), was used to titrate enzyme fluorescence, the results obtained indicated that an extremely tight binding occurred (Figure 8A). Methotrexate also quenched the enzyme–NADPH complex fluorescence measured at 445 mμ when activated at either 290 or 350 mμ (Figure 8B and Figure 8C). Since the dissociation of the complexes with methotrexate was so slight, the equivalence point, as determined by the definite inflection of the curve, could be used to determine accurately the enzyme concentration. If 1 mole of the inhibitor binds to 1 mole of enzyme, then the enzyme concentration was the same as that determined from methotrexate titration of enzyme activity, *i.e.*, 5.6×10^{-7} M.

Although methotrexate quenched the fluorescence of the complex, simple displacement of the coenzyme by the inhibitor did not appear likely since the level of fluorescence was reduced to a value lower than would have been expected from the amount of NADPH present. The results of the experiment illustrated in Figure 9 support an alternate explanation; namely, that methotrexate, NADPH, and the enzyme form a nonfluorescent ternary complex. In this experiment three different concentrations of enzyme–methotrexate complex were titrated with NADPH. The data form a family of parallel lines that were displaced along the abscissa to a degree dependent on the concentration of the enzyme–methotrexate complex; the intercepts of the abscissa actually indicate the enzyme concentration in each titration. These results show that the added coenzyme was tightly bound, up to the point of a 1:1 stoichiometry, to the enzyme–methotrexate complex. Further additions of NADPH resulted in an essentially linear increase in fluorescence attributable to free coenzyme.

Evidence for an Enzyme–Coenzyme–Triampterene Complex. It was shown in Figure 6 that a binary complex was formed between the enzyme and triampterene resulting in quenching of the fluorescence of the enzyme. Advantage has been taken of the strong fluorescence of triampterene (activation maximum 380 mμ, emission maximum 445 mμ) to demonstrate that this inhibitor also forms a ternary complex with the enzyme and NADPH or NADP$^+$. The effect of FH_2 reductase on the fluorescence of triampterene in the presence of the coenzymes and in their absence is illustrated in Figure 10. The enzyme alone caused a decrease in the fluorescence that was in accord with the degree of association of triampterene and the free enzyme shown in Figure 6. In the presence of either NADPH or NADP$^+$, however, the enzyme was much more effective in quenching the fluorescence of triampterene. Since the coenzymes neither quenched the fluorescence of triampterene nor fluoresced measurably under these conditions, these

[4] In the equations used to describe the theoretical curves in Figures 6, 7, 10, and 11, it should be noted that the values of $F°$ and F used were not the absolute value indicated by the ordinate of the figures but rather $F° - F_s$ and $F - F_s$, where F_s is the fluorescence at the point of saturation with the respective titrants.

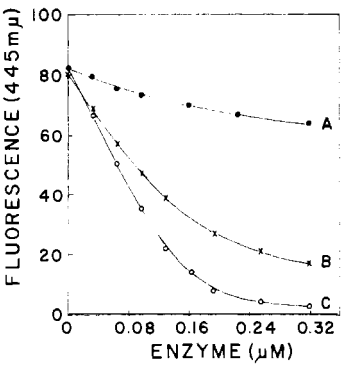

FIGURE 10: Demonstration of a ternary complex between FH_2 reductase, NADPH, and triampterene. Activation was at 380 mμ and the emission intensity was measured at 445 mμ. Curve A represents the effect of enzyme alone on the fluorescence of triampterene. Curves B and C illustrate the effect of 7.3×10^{-6} M $NADP^+$ and 1.4×10^{-6} M NADPH, respectively, on the titration of triampterene fluorescence with FH_2 reductase. The triampterene concentration was 1.75×10^{-7} M in each case. The buffer was the same as in Figure 1. The meter multiplier was set at 0.3.

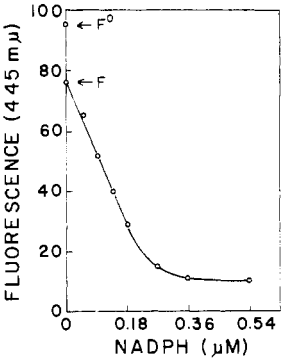

FIGURE 11: Determination of the K_D value for NADPH dissociation from the ternary complex. Activation was at 380 mμ and emission intensity was measured at 445 mμ. The concentration of FH_2 reductase was 2.8×10^{-7} M and that of triampterene was 2.1×10^{-7} M. $F°$ indicates the fluorescence of triampterene alone, while F indicates the fluorescence of the mixture of enzyme and triampterene. The buffer was the same as in Figure 1. The meter multiplier was set at 0.3.

results indicated that a complex between enzyme, coenzyme, and triampterene was formed. Curve C in Figure 10 was plotted in a manner similar to that described for the data in Figure 6 for $K_D = 5 \times 10^{-9}$ M; curve B was plotted in a like manner based on a K_D of 3×10^{-8} M. The titrations were carried out in the presence of excess coenzyme and the estimation of the dissociation curves was based on the assumption of a 1:1:1 stoichiometry for the ternary complex.[5] These observations indicated that triampterene was bound approximately 60 times more tightly in the ternary complex than to the free enzyme.

When solutions of FH_2 reductase and triampterene were titrated with either NADPH (Figure 11) or $NADP^+$ (not illustrated) the results indicated that the coenzymes were also bound more tightly in the ternary complex than to the free enzyme. The K_D value for NADPH determined[6] from the data in Figure 11 was 2×10^{-9} M and is approximately 25 times less than the K_D value for dissociation from the binary complex with free enzyme. Thus, the formation of the ternary complex caused a mutual stabilization of binding of both the coenzyme and inhibitor. When methotrexate was added to a solution of the above-defined ternary complex, essentially complete regeneration of the fluorescence of triampterene was observed, indicating that a displacement of the triampterene from the complex by the more strongly bound inhibitor, methotrexate, had occurred.

Discussion

Highly purified preparations of FH_2 reductase have been used in the studies reported here. The enzyme concentration was based on the titration of enzyme activity with a highly specific (Schrecker and Huennekens, 1964), stoichiometric (Werkheiser, 1961) inhibitor. The accuracy of the method depends in part on the assumption that 1 mole of inhibition binds per mole of enzyme and that all of the methotrexate molecules and all of the enzyme molecules are fully reactive at the pH used for the titration, *i.e.*, pH 5.9. The calculation of enzyme purity further depends on the assumption that the molecular weight of the enzyme as determined by gel filtration on Sephadex is accurate.

The specificity of binding indicated by these studies further supports the assumption that the interactions observed were indeed between FH_2 reductase and its substrates, coenzymes, and inhibitors. At this time it is not apparent whether the lack of complete quenching of enzyme fluorescence by the various agents (Figures 6, 7) was simply a property of the enzyme (see Velick,

[5] The calculations for the dissociation curves in Figure 10 were based on the assumption that once added, the enzyme existed almost entirely in the form of the enzyme–coenzyme complex (EA) or as the ternary complex (EAI). Since $K_D = (EA)(I)/(EAI)$, and (I) is proportional to fluorescence (F), it was possible to calculate the dissociation curves in terms of (E°) (enzyme added) for various values of F from the relationship $(EA°) = K_D(F°/F - 1) + (I°)(1 - F/F°)$, where $F°$ is the initial fluorescence, (I°) is the initial concentration of triampterene, and (EA°) = (E°).

[6] Since $K_D = (EI)(A)/(EAI)$ where (E) is enzyme, (A) is coenzyme, and (I) is triampterene, at one-half saturation (EAI) = (I°)/2, where (I°) is the initial concentration of triampterene. Also, (I) = $(I°)(F/F°)$, where $F°$ and F are the initial fluorescence and the fluorescence at one-half saturation, respectively. Since (EI) = (I°) − (EAI) − (I) and (A) = (A°) − (EAI), where (A°) is the coenzyme added, the K_D value for NADPH can be calculated in terms of F, (A°), and (I°). The assumption has been made that the concentration of (EA) is insignificant under the experimental conditions.

1958; McKay and Kaplan, 1964) or a contribution to protein fluorescence due to an impurity. It should be noted, however, that when methotrexate was added to a solution of 90% pure FH_2 reductase, 90% of the protein fluorescence was quenched (Figure 8A).

When solutions of FH_2 reductase and NADPH were mixed, alterations occurred in the fluorescence emission spectra of both enzyme and coenzyme that indicated the formation of a complex. The observations reported here are similar to those of Velick (1958) and Winer *et al.* (1959) for lactate dehydrogenase and Theorell and his co-workers for alcohol dehydrogenase (Boyer and Theorell, 1956; Theorell and McKinley-McKee, 1961). The data obtained from the titration experiments described in this report have allowed the estimation of the dissociation constants for the binary complexes formed between the enzyme and NADPH, $NADP^+$, NADH, FH_2, folate, and triampterene. These experiments provide direct evidence that the enzyme forms complexes with either the coenzyme or the substrate in the absence of the other coreactant at concentrations similar to or lower than the values obtained for kinetic constants. Table I provides a summary of the various dissociation constants determined from these studies and lists for comparison certain kinetic constants determined in this laboratory for the L1210R FH_2 reductase.

The enzymatic mechanism of most dehydrogenases so far examined has been shown to be of the type described by Cleland (1963) as Ordered Bi Bi, *i.e.*, an ordered addition of reactants, with the coenzyme binding first, then ordered expulsion of products, with the coenzyme leaving last. Evidence has accumulated that such a mechanism applies to lactate dehydrogenase (Takenaka and Schwert, 1956), liver and yeast alcohol dehydrogenases (Baker, 1962; Wratten and Cleland, 1963), malate dehydrogenase (Raval and Wolfe, 1962), and ribitol dehydrogenase (Fromm and Nelson, 1962). The mechanism implies that the substrate forms kinetically significant complexes only with the enzyme–coenzyme complex. The observation that FH_2 forms a binary complex with the free enzyme characterized by a dissociation constant (2.5×10^{-7} M) similar to its K_m value (4×10^{-7} M) is consistent with a kinetic mechanism characterized by random addition of substrates.

Hakala (1965) in a preliminary communication has suggested that FH_2 reductase forms binary complexes in random order with its substrates and coenzymes. This conclusion was based on the observation that NADPH, $NADP^+$, or FH_2 protected the enzyme from inactivation by treatment with a variety of agents. We also have observed that both coenzyme forms, as well as FH_2, protect the enzyme from inactivation by either moderate heat or by treatment with urea and that the rate of reaction of *p*-mercuribenzoate with the enzyme is decreased when either NADPH or FH_2 is present.[3] These observations, as well as the findings from the fluorometric studies presented here, indicate that a kinetic mechanism for FH_2 reductase must be compatible with the binding of both substrate and coenzyme to the free enzyme.

The strong fluorescence of the pteridine analog, triampterene, was quenched in the presence of FH_2 reductase when either NADPH or $NADP^+$ also was present. Since the coenzymes alone did not quench the fluorescence and since the quenching by the enzyme alone was only a small fraction of that observed in the presence of all three components, these findings indicated that a ternary complex was formed. The formation of ternary complexes between coenzyme, inhibitor, and other dehydrogenases has been reported by Winer and Schwert (1959), Theorell and Langan (1960), and Theorell and McKinley-McKee (1961). The latter authors reported that liver alcohol dehydrogenase formed ternary complexes in the presence of NADH or NAD^+ and certain inhibitors. They were able to determine the dissociation constants of the various binary and ternary complexes from changes in fluorescence properties. They concluded that a mutual stabilization of binding occurred from the formation of the enzyme–coenzyme–inhibitor complex that resulted in 20- to 70-fold increases in the affinity of the enzyme for both the coenzyme and inhibitor over the affinities measured in binary complexes. Our studies with FH_2 reductase have been interpreted in a similar manner. As can be seen in Table I, the K_D values for NADPH and triampterene dissociation from the ternary complex are 25–60 times less than those determined for dissociation of the various binary complexes.

The stoichiometric binding of methotrexate to either the free enzyme or the enzyme–NADPH complex provided independent confirmation of the titrating type of inhibition exhibited by this analog. Because of the extremely tight binding of methotrexate in both binary and ternary complexes, the conditions employed in these studies did not disclose differences in the respective dissociation constants. The finding that the inhibitor triampterene is bound more tightly in ternary complexes than to the enzyme alone, suggests, however, that this may also be the case with methotrexate. In support of this concept, we have found that when varying concentrations of the enzyme–methotrexate complex were titrated with NADPH, complete quenching of the NADPH fluorescence was observed until a stoichiometry of 1:1:1 was obtained; further additions of NADPH caused an essentially linear increase in fluorescence (see Figure 9). The sharp inflection at the molar equivalence point was indicative of extremely tight binding of the coenzyme in the ternary complex.

These observations suggest that the inhibition by triampterene and methotrexate observed in kinetic experiments is attributable to binding to the enzyme–NADPH complex. These findings may be of importance in gaining an understanding of the nature of the binding of methotrexate, in particular of the dependence of inhibition on pH.

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