Kinetic and Chlorine-35 Nuclear Magnetic Resonance Studies of the Effect of Chloride on the Properties of Chicken Liver Dihydrofolate Reductase†

S. Subramanian,* H. Shindo,‡ and Bernard T. Kaufman

ABSTRACT: The reduction of dihydrofolate to tetrahydrofolate catalyzed by dihydrofolate reductase from chicken liver has been studied in the presence and absence of sodium chloride. The reaction using the coenzyme NADPH was activated by sodium chloride, with a maximum effect (~40%) in the range of 0.2–0.6 M, with declining effects beyond that concentration. Comparable activations were also found with bromide and iodide. When NADH was used as the coenzyme, the reaction was increasingly inhibited as a function of salt concentration. Such salt effects do not shift the activity–pH profiles except for the activation/inhibition effects. The binding of the chloride anion to the enzyme was studied by chlorine-35 nuclear magnetic relaxation. The longitudinal relaxation rate ($1/T_1$) of ^{35}Cl in the presence of the enzyme was increased over that in the absence of the enzyme, indicating binding of chloride to the enzyme. This rate decreased when $NADP^+$ or NADPH was added to the enzyme but was practically unaffected by the addition of NADH. Addition of dihydrofolate or methotrexate to the enzyme increased the relaxation rate of chloride slightly while addition of Cibacron Blue F3GA did not affect it. Methotrexate addition to the enzyme–NADPH or enzyme–$NADP^+$ complex did not affect the relaxation rate of chloride; however, it decreased the ^{35}Cl relaxation rate when added to the enzyme–NADH complex. These results indicate that chloride and NADP(H) bind to the enzyme in a competitive and mutually exclusive manner and that chloride and NADH may be bound to the enzyme simultaneously except in the presence of methotrexate when NADH displaces bound chloride. The activations by salt of the catalytic reactions using NADPH may be due to the loosening of NADP(H) binding to the enzyme by salt, thereby facilitating the faster release of products. However, chloride binds to the enzyme in the presence of NADH and probably prevents the effective formation of the Michaelis complex, thereby causing inhibition. A similar behavior is also found with NADPH at high salt concentration (>1 M) when the reaction is inhibited by salt. An attempt is made to characterize the residues on the enzyme to which chloride may bind.

The modulating effects of salts on the activities of several enzymes have been investigated, and activation and inhibition patterns have been noted with both cations and anions. While the effects of the ions on the structure and stability of proteins may be due to general solvent structural changes as well as specific binding of the ions to proteins (von Hippel & Schleich, 1969), the effects of salts on enzyme activities are generally recognized to arise from specific binding and generally occur at lower concentrations than needed for manifestations of structural changes (Carlson & Graves, 1976; Nakashima & Tsuboi, 1976). The effect of anions (independent of the countercation) on the activities of dehydrogenases using pyridine nucleotides as coenzymes (Corman & Kaplan, 1967; Coleman & Weiner, 1973; Erickson & Mathews, 1973) is highlighted by the fact that the anions could compete with the negatively charged phosphate groups of the coenzymes and thereby regulate the enzyme activity.

The effect of salts on the activity of dihydrofolate reductase (EC 1.5.1.3) from a number of sources has been studied, and while the enzymes from bovine liver and L1210 are reported to be activated by salt (Reyes & Huennekens, 1967; Peterson et al., 1975), those from *Escherichia coli* and bacteriophage T4 are inhibited by salt (Erickson & Mathews, 1973). Such conflicting reports make it impossible to present a unified mechanism of the action of salt on the enzyme. It has been recognized, however, that the salt effects noted above are solely due to the anions.

It is necessary to delineate the details of the anion interactions with the enzyme in order to understand whether the activation/inhibition phenomena are a result of conformational changes in the enzyme and/or the coenzymes or due to specific and mutual accommodation or exclusion between the anions and coenzymes for binding sites on the enzyme. Nuclear magnetic resonance experiments can shed light on the nature of binding of anions to enzymes and proteins (Forsen & Lindman, 1978) at the molecular level regarding the nature and locus of the binding sites (Andersson et al., 1979).

In this communication, we describe ^{35}Cl magnetic resonance relaxation studies in the presence of dihydrofolate reductase from chicken liver with and without coenzymes, substrates and substrate-analogue inhibitors. We also report the effect of chloride on the kinetic properties of the enzyme in order to elucidate the mechanism of salt action.

Experimental Procedures

Materials. Chicken liver dihydrofolate reductase was isolated and purified by the procedure of Kaufman & Kemerer (1977). Folic acid, $NADP^+$, NADPH, and NADH were purchased from Calbiochem or Sigma. Methotrexate was a gift of A. Mead (National Cancer Institute). Dihydrofolate was prepared from folic acid by dithionite reduction (Blakley, 1960). The concentrations of methotrexate and dihydrofolate were determined (Subramanian & Kaufman, 1978) by using standard extinction coefficients. Cibacron Blue F3GA was obtained from Sigma. Cibacron blue concentrations were determined by using an extinction coefficient of 13 600 M^{-1} cm^{-1} at 610 nm (Thompson & Stellwagen, 1976).

Methods. The enzyme concentration was determined by using an extinction coefficient, ϵ_{280nm} = 28 970 M^{-1} cm^{-1}, or from activity measurements. Chicken liver dihydrofolate reductase has an activity of 15 units/mg of enzyme (Subra-

† From the Laboratory of Nutrition and Endocrinology (S.S. and B.T.K.) and the Laboratory of Chemical Physics (H.S.), National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205. *Received November 14, 1980.*

‡ Present address: Tokyo College of Pharmacy, Horinouchi, Hachioji, Tokyo, Japan.

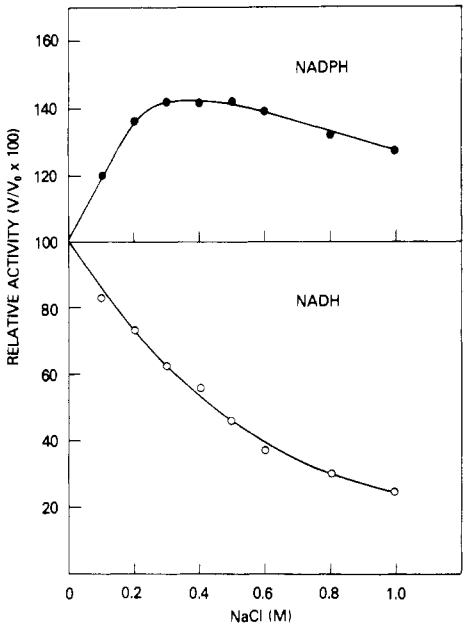

FIGURE 1: The initial rates of the reduction of dihydrofolate by chicken liver dihydrofolate reductase as a function of added sodium chloride concentration. V is the velocity in the presence and V_0 the velocity in the absence of NaCl. For the reaction using NADPH as the coenzyme, the concentration of NADPH was 100 μM and that of enzyme 0.11 μM; for the reaction using NADH, the NADH concentration was 186 μM and that of enzyme 1.07 μM. In both cases, dihydrofolate was present at a final concentration of 60 μM. Experimental conditions: 1-mL cuvettes were used; temperature 28 °C; pH 7.4 with 50 mM potassium phosphate buffer.

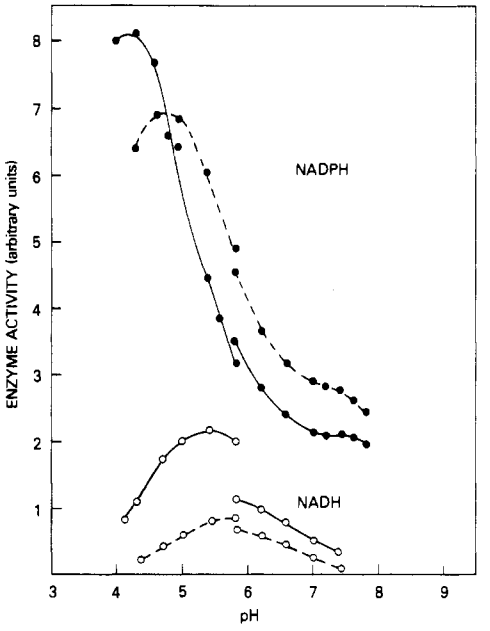

FIGURE 2: Effect of 0.2 M NaCl on the pH–activity profile of chicken liver dihydrofolate reductase using either NADPH or NADH with dihydrofolate as substrate. The standard reductase assay as described in Figure 1 was performed at the various pH values. In a final volume of 3 mL, the buffer concentration was 100 mM with acetate at pH 5.8 and below and phosphate at pH 5.8 and above. The solid line is the control, and the dashed line denotes the presence of 0.2 M NaCl. Temperature was 28 °C.

manian & Kaufman, 1978) where one unit of activity is defined as that catalyzing the reaction of 1 μmol of NADPH and dihydrofolate per min under the experimental conditions. This corresponds to a ΔA_{340nm} equal to 185 min^{-1} (mg of enzyme)$^{-1}$. The assays were made according to Mathews et al. (1963) at 28 °C by using a Gilford (Model 222A) spectrophotometer.

Kinetics. Kinetic measurements were made by using a Cary (Model 219) spectrophotometer thermostated at 25 °C. The initial rates were derived from the changes in absorbance at 340 nm by using a value of 12 300 as the combined molar extinction coefficient for NADPH and dihydrofolate (Hillcoat et al., 1967). The K_m values were determined according to standard procedures.

^{35}Cl NMR Relaxation Experiments. ^{35}Cl NMR measurements were made at 26.5 MHz on a home-built spectrometer with a Bruker magnet (6.4 T) and a Nicolet 1180 computer. The inversion-recovery method with a 180°–τ–90° pulse sequence was applied for measurements of longitudinal relaxation times, T_1. T_1 values were obtained by least-squares analysis of the data; the imprecision in T_1 was less than 3% for all sets of experiments. Temperature was regulated by controlling the flow of cooled nitrogen and was directly measured by a calibrated thermometer (Doric Model 400) with a very thin thermocouple. The temperature for the T_1 measurements was 10 ± 0.5 °C in all experiments. Sodium chloride (0.1 M) in the presence of 0.1 M potassium phosphate at pH 7.4 was used in all ^{35}Cl relaxation measurements.

Results

The relative activities of the reaction catalyzed by chicken liver dihydrofolate reductase in the presence and absence of several concentrations of NaCl by using either NADPH or NADH as the coenzyme are shown in Figure 1. With NADPH as the coenzyme, the reaction is activated by salt, with a maximum activation of 40% in the range 0.2–0.6 M NaCl. The ratio of V/V_0 becomes unity again at around 1.3 M NaCl, beyond which the reaction is inhibited. KCl and NaCl have the same effects, and, hence, activation is due to the anion. At comparable concentrations, the effect of the salts varies as NaI > NaBr > NaCl. For the bromide and iodide, the inhibition also starts at lower concentrations than that of chloride. When NADH is used as the coenzyme, the reaction is inhibited monotonically as a function of chloride concentration.

Table I: Effect of Chloride Concentration on the Apparent K_m for NADPH and NADH in the Dihydrofolate Reductase Catalytic Reaction[a]

NaCl (M)	K_m (NADPH) (μM)	K_m (NADH) (μM)
0	0.63	44
0.2	1.72	56
0.4	4.15	62
0.6	8.68	85

[a] Temperature 25 °C; pH 7.4 with 50 mM potassium phosphate buffer.

In Figure 2 are shown the pH–activity profiles of the catalytic reaction. The presence of 0.2 M salt activates the reaction by using NADPH as the coenzyme at all pH values from 4.5 to about 8.0, and only slight shifts in the pH optima are evident. Similarly, the inhibition of the NADH reaction by salt is also not accompanied by any shift in pH optima in the range 4–8. Notice that the inhibition by salt is greater in the pH range 4–6 where acetate buffer is used than in the pH range 6–8 where phosphate buffer is used.

The approximate K_m values for the coenzymes in the reductase reaction in the absence of salt and in the presence of 0.2, 0.4, and 0.6 M NaCl concentrations are given in Table I. From 0 to 0.6 M NaCl, the K_m for NADPH increases about 15-fold while the K_m for NADH increases only 2-fold.

Solutions of chicken liver dihydrofolate reductase and chloride ions show enhanced longitudinal relaxation of ^{35}Cl compared to the chloride ions alone. For example, at 10 °C,

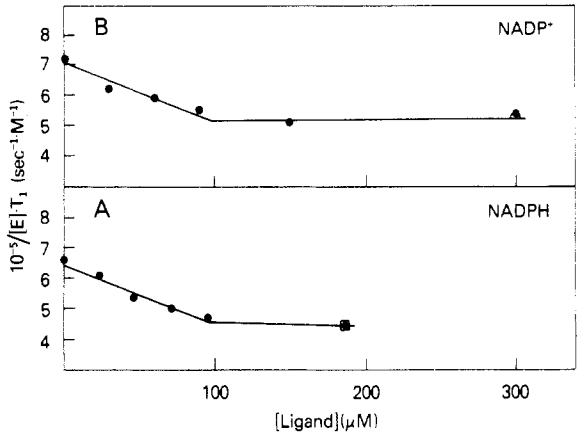

FIGURE 3: Longitudinal ^{35}Cl relaxation rate divided by the enzyme concentration, [E] (chicken liver dihydrofolate reductase), as a function of added ligand concentration (NADPH or $NADP^+$). Enzyme concentration was 108 μM for NADPH titration and 87.5 μM for $NADP^+$ titration. The open squares coinciding with the filled circles at the highest NADP(H) concentration represent the addition of (A) 101 μM and (B) 98 μM methotrexate to the enzyme–chloride–ligand complex. The longitudinal relaxation times, T_1, were measured at 10 °C; pH was 7.4; chloride concentration was 0.1 M.

a solution of 0.1 M chloride in the buffer solution at pH 7.4 has a longitudinal relaxation time, T_1, of 25 ± 0.2 ms. With addition of enzyme, the relaxation time diminishes (rate increases) as a function of the enzyme concentration. The relaxation time is about 15 ms at an enzyme concentration of 100 μM and 13 ms at 200 μM enzyme. For purposes of discussion, the rates of relaxation ($1/T_1$) rather than the times (T_1) will be used, and for the sake of comparison, the rates are all normalized with respect to the enzyme concentration since different enzyme concentrations were used in different measurements. The enhanced relaxation rate of ^{35}Cl in the presence of the enzyme decreases as a function of added ligand ($NADP^+$ or NADPH) until a stoichiometric ratio of unity for the enzyme:ligand complex is reached with no further changes in the relaxation rate for further additions of the ligands (Figure 3). For example, T_1 for chloride in the presence of 108 μM enzyme is 14.1 ± 0.2 ms which changes to 19.7 ± 0.3 ms upon addition of 95 μM NADPH and 20.3 ± 0.3 ms upon addition of 186 μM NADPH. $NADP^+$ produces an almost indentical effect in the chloride relaxation. Addition of about 100 μM methotrexate, a tight-binding substrate analogue of the enzyme, to the solution containing the enzyme, chloride, and saturating levels of $NADP^+$ or NADPH, results in no further change in the relaxation rate of chloride. The relaxation rate of chloride in the presence of the enzyme and $NADP^+$ or NADPH with or without methotrexate never regains the value of free chloride in the absence of the enzyme, indicating possible extraneous chloride binding sites other than the active site on the enzyme and/or a general viscosity effect.

The titration of the enzyme–chloride complex with NADH, unlike $NADP^+$ or NADPH, does not result in a decrease of the relaxation rate of ^{35}Cl. If anything, there is a slight increase in the rate as NADH is added (Figure 4A). However, when methotrexate (80 μM) is added to the enzyme–chloride–NADH complex, the rate decreases. (T_1 for ^{35}Cl in the presence of 89 μM enzyme is 14.3 ± 0.1 ms; for the enzyme–chloride–NADH complex at a concentration of 89 μM enzyme and 413 μM NADH, T_1 is 13.6 ± 0.1 ms, which changes to 16.8 ± 0.1 ms upon addition of 80 μM methotrexate.) Conversely, there is no change in the relaxation rate for an addition of 80 μM methotrexate to the enzyme–chloride complex, but when this enzyme–chloride–methotrexate complex is titrated with NADH, the rate decreases (Figure 4B).

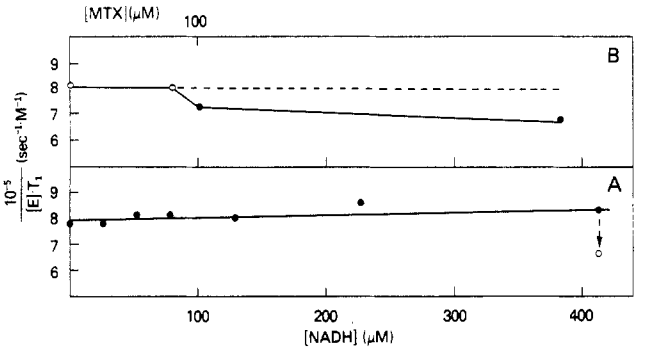

FIGURE 4: Longitudinal ^{35}Cl relaxation rate divided by enzyme concentration, [E], for titration of chicken liver dihydrofolate reductase–chloride solution with NADH (A). The open circle in the bottom frame denotes addition of 85 μM methotrexate to the enzyme–chloride–NADH complex. (b) Methotrexate, 80 μM, was first added (open circle) to the enzyme–chloride complex and then titrated with NADH (filled circles). The enzyme concentration was 89 μM in both experiments. Temperature, 10 °C, pH 7.4; chloride concentration 0.1 M. The dashed line in the top frame is an extrapolated line for titration with methotrexate.

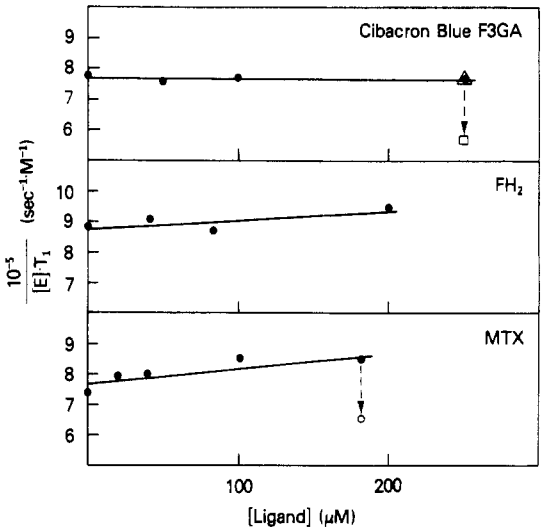

FIGURE 5: Longitudinal relaxation rate of ^{35}Cl divided by concentration of enzyme, [E], for the titration of chicken liver dihydrofolate reductase–chloride solution with various ligands. Temperature 10 °C, pH 7.4; chloride concentration 0.1 M. (Bottom frame) Enzyme concentration 89 μM; titration with methotrexate (MTX). The open circle denotes the addition of 164 μM NADPH to the enzyme–chloride–methotrexate complex. (Middle frame) Enzyme concentration 89 μM; titration with dihydrofolate (FH_2). (Top frame) Enzyme concentration 87.5 μM; titration with the blue dye Cibacron Blue F 3GA. The open triangle represents the addition of 200 μM methotrexate to the enzyme–chloride–complex. The open square denotes a subsequent addition of 111 μM NADPH.

Thus the ^{35}Cl relaxation rate decreases only in the enzyme–methotrexate–NADH–chloride quaternary complex and not in enzyme–NADH–chloride or enzyme–methotrexate–chloride ternaries relative to the enzyme–chloride complex.

In Figure 5 the effects of methotrexate (MTX), dihydrofolate (FH_2), and Cibracron Blue F3GA on the relaxation rate of ^{35}Cl in the presence of the enzyme are shown. Addition of methotrexate or dihydrofolate to the enzyme–chloride complex results in small *increases* in the rate while the addition of Cibacron Blue does not affect the rate at all. Addition of methotrexate to the enzyme–chloride–Cibacron Blue complex does not affect the rate, but subsequent addition of NADPH decreases the rate. [T_1 for chloride with enzyme alone is 14.6 ± 0.2 ms; T_1 for chloride in enzyme–chloride–blue dye is 14.8 ± 0.1 ms, which is unchanged upon addition of 200 μM methotrexate but changes to 20.0 ± 0.1 ms upon subsequent

addition of 111 μM NADPH (Figure 5, top frame).]

Discussion

The destabilization of protein structure and the modulation of enzyme activities by neutral salts have been widely observed. The effectiveness of anions in bringing about these changes in a large number of macromolecules is described by the anionic Hofmeister series. The effect of anions on enzyme activities of dehydrogenases has also been widely studied. The coenzyme NAD(P) has two or three negative charges which specifically interact with positively charged amino acid residues on the enzyme. Extraneous anions could compete (for these positively charged residues) with the phosphate/pyrophosphate moieties of the coenzyme. The positive charge on the nicotinamide ring of the oxidized coenzymes does not seem to play any active role in binding; the effect of the positive charge is, in fact, to lessen the affinity of the oxidized coenzyme relative to that of the reduced coenzyme in most dehydrogenases, and thus cationic effects are much less evident than anionic effects.

The effect of chloride on the dihydrofolate reduction catalyzed by chicken liver dihydrofolate reductase is characterized by activation when NADPH is used as the coenzyme and inhibition with NADH as the coenzyme. Previous studies with bacterial (Erickson & Mathews, 1973) and mammalian enzymes (Reyes & Huennekens, 1967; Peterson et al., 1975) used only NADPH. In the reaction catalyzed by the bovine liver enzyme (Peterson et al., 1975), the pH optimum for the reaction was shifted from 4.8 to 6.2 in the presence of 0.6 M KCl. With the chicken liver enzyme, the pH optima change only slightly in the presence of salt, signifying the absence of major mechanistic changes in the kinetics of the reaction. The opposite salt effects noted with NADPH and NADH may signify differences in the mode of binding of the coenzyme to the enzyme or subtle changes in conformational states of the enzyme in the presence of salt and either coenzyme.

One noteworthy feature of the salt effect with NADH is that the inhibitory effect of chloride is much higher in the pH range 4–6 where acetate is used as the buffer salt. If acetate does not bind to the enzyme as well as phosphate, the competition of the chloride ion with the buffer salt will be more manifest with the acetate buffer in producing the inhibitory effect. Such buffer salt–anionic competition is probably not significant for the NADPH reaction. The apparent K_m values for NADPH and NADH are 0.63 and 44 μM, respectively, in the absence of added salt. With 0.6 M chloride, these values change to 8.68 and 85 μM, respectively. This indicates the relative insensitivity of NADH binding affinity to the enzyme to the presence of chloride. However, the NADH reaction is inhibited by chloride, while with NADPH, whose K_m is affected greatly by salt, the reaction is activated by chloride. One reason for this dichotomy could be the 2′-phosphate moiety. If chloride competes effectively with the 2′-phosphate for an enzyme subsite, it could result in a substantial loosening of NADPH from the enzyme, and this could in turn be responsible for the activation of the reaction if product release is the rate-limiting step. On the other hand, with NADH, if the binding of the substrates is weakened by the presence of chloride, although only slightly, inhibitory effects could set in due to the decreased affinity of the reactant(s).

The decreased longitudinal relaxation rate for ^{35}Cl in the presence of the enzyme upon addition of $NADP^+$ or NADPH is due to competition with chloride. Since $NADP^+$ and NADPH have binding constants of 2.6×10^5 and 3.22×10^6 M^{-1}, respectively, to the chicken liver enzyme (Subramanian & Kaufman, 1978), they must both bind much more strongly than chloride and should release chloride ions stoichiometrically. This can account for the linear ^{35}Cl relaxation rate decrease with added $NADP^+$ or NADPH and the essentially constant value beyond a stoichiometric 1:1 ratio of enzyme to coenzyme (Figure 3). The fact that the addition of equimolar methotrexate to the enzyme–chloride–NADP(H) complex does not change the relaxation rate any further indicates the absence of chloride binding at the methotrexate binding site. Such chloride release upon coenzyme binding has also been noticed with liver alcohol dehydrogenase (Andersson et al., 1979); the longitudinal relaxation rate of ^{35}Cl in the presence of rabbit muscle lactic dehydrogenase was unaffected by addition of NADH and decreased only when oxamate (a substrate analogue) was added (Bull et al., 1976). When binding to lactic dehydrogenase, chloride competes with the anionic substrate rather than the coenzyme.

The three-dimensional structure of dihydrofolate reductase from *Lactobacillus casei* has been determined at 2.5-Å resolution for the enzyme–methotrexate–NADPH complex (Matthews et al., 1978), and the stereochemistry of the NADPH binding and the molecular interactions have been described (Matthews et. al., 1979). The 2′-phosphate and pyrophosphate of NADPH are involved in charge interactions with Arg-43 and Arg-44, respectively, of the *L. casei* enzyme. In the chicken liver enzyme, based on sequence homology (Kumar et al., 1980), the corresponding residues involved in electrostatic interactions would be Lys-54 and Lys-55. If in the absence of coenzyme chloride ions interact with these residues on the enzyme, then upon addition of NADP(H) these chloride ions would be released, resulting in a relaxation rate decrease. The fact that the rate decreases up to a 1:1 stoichiometric ratio of the enzyme to coenzyme and then remains constant argues for such a direct displacement of chloride even though displacement mediated through a conformational change cannot be ruled out.

NADH, unlike NADPH, upon addition to the chicken liver dihydrofolate reductase containing the chloride does not cause a ^{35}Cl relaxation rate decrease (Figure 4A). Clearly, NADH addition does not seem to cause a release of bound chloride. The slight enhancement in the relaxation rate is similar to that seen for the T_1 of ^{35}Cl with rabbit muscle lactic dehydrogenase (Bull et al., 1976). It is likely that an increased correlation time for the bound chloride upon addition of NADH is responsible for the slight enhancement of the relaxation rate. The lack of 2′-phosphate in NADH is presumably one factor in its not being able to cause a change in the chloride relaxation. However, the increase in T_1 upon addition of methotrexate to enzyme–chloride–NADH complex is not seen with enzyme–NADPH complex or with methotrexate alone when added to the enzyme–chloride complex. Methotrexate, by itself, causes a slight enhancement in the chloride relaxation rate as does dihydrofolate, the natural substrate, when added to the enzyme–chloride complex. Whether methotrexate is added to enzyme–chloride–NADH complex or NADH is added to enzyme–chloride–methotrexate complex, the effect is the same, viz., a ^{35}Cl relaxation rate decrease similar to that seen with NADPH addition to enzyme–chloride complex but of reduced magnitude. These results could be explained if we assume that when NADH binds to chicken liver dihydrofolate reductase, the mode of binding is different from that of NADPH.

It has been noted (Matthews et al., 1979) that the conformation for NADPH when bound to dihydrofolate reductase and that for NAD^+ when bound to lactate-, malate-, and glyceraldehyde-3-phosphate dehydrogenases are quite different in terms of the $C6_a$–$C2_n$ distance and also the dihedral angle

between the adenine and the nicotinamide planes. If NADH is bound to dihydrofolate reductase in a mode similar to that of NAD^+ in lactic dehydrogenase, it is possible that NADH and chloride could simultaneously bind to the enzyme and the relaxation rate of chloride would be unaffected, as is the case with lactic dehydrogenase (Bull et al., 1976). Upon addition of methotrexate to the enzyme–chloride–NADH complex, either a conformational change in the enzyme or a change in the mode of binding of NADH could cause a displacement of chloride and thus a relaxation rate decrease.

Dihydrofolate, the natural substrate for the enzyme, and methotrexate, the substrate-analogue inhibitor which is known to bind at the dihydrofolate binding site on the enzyme, produce a slight enhancement in the chloride longitudinal relaxation rate (Figure 5). It is obvious that methotrexate and dihydrofolate do not displace any bound chloride. The decrease in relaxation rate observed when NADPH is added to the enzyme–chloride–methotrexate complex is comparable to that obtained when NADPH alone is added to the enzyme–chloride complex. These observations suggest that chloride binding and its displacement occur only at the NADPH binding site of the enzyme and that a change in the mode of binding of NADH to the enzyme upon addition of methotrexate may occur subsequently, releasing bound chloride.

Cibacron Blue, a dye used widely in the affinity column material Blue Dextran–Sepharose, when added to the enzyme–chloride complex does not change the chloride relaxation rate. Thus, like methotrexate, the blue dye has no effect on chloride binding; it is known that the blue dye binds at the methotrexate site of the enzyme (Subramanian & Kaufman, 1980). Replacement of the dye with methotrexate does not affect the chloride relaxation, but subsequent addition of NADPH decreases the chloride relaxation, which is in accord with chloride displacement from the NADPH site only.

It is clearly seen that $NADP^+$ and NADPH compete with chloride ions for binding sites on the enzyme and that NADPH displaces chloride and chloride raises the K_m for NADPH. It is this property that makes chloride activate the NADPH reaction by loosening the tight binding of NADPH and its oxidation product $NADP^+$. The binding of NADH and chloride to the enzyme are inclusive, and chloride raises the K_m for NADH only slightly. Since the binding of NADH to the enzyme is 100-fold weaker than that of NADPH, bound chloride may interfere with an effective and productive binding of NADH, thereby causing inhibition of the reaction by using NADH as the coenzyme. This study thus raises the possibility of a differential mode of binding of NADH and NADPH to chicken liver dihydrofolate reductase.

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