Biochemistry 1981, 20, 464-467

Effects of Halides on Reduced Nicotinamide Adenine Dinucleotide Binding Properties and Catalytic Activity of Beef Heart Lactate Dehydrogenase[†]

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ABSTRACT: Beef heart lactate dehydrogenase has anion binding sites with selective affinities for fluoride and chloride. Fluoride competitively inhibits the catalytic activity of the enzyme and appears in a ternary enzyme-reduced nicotinamide adenine dinucleotide (NADH)-fluoride complex detectable in fluorescence and circular dichroism measurements. The presence of fluoride augments NADH binding, with a free energy of stabilization of -0.8 kcal/mol. NADH and chloride are strongly antagonistic, in fact, almost mutually exclusive or competitive, in their interaction with beef heart lactate dehydrogenase. In addition, the Hill coefficient for NADH binding undergoes a small but repeatable decline, reaching a minimum value of 0.75-0.8 at physiological NaCl concentrations. Dilution experiments showed that NADH binding in the presence of NaCl is independent of enzyme concentration, demonstrating that the chloride sensitivity is not linked to reversible dissociation of the enzyme. The NADH binding equilibria determined in NaCl, KCl, or CsCl are identical. The minimal effects of chloride on the fluorescence and circular dichroism spectra of the bound NADH suggest that it binds primarily at sites other than the one occupied by fluoride.

X-ray diffraction studies show that lactate dehydrogenase has at least two types of anion binding sites in addition to the pyridine nucleotide site (Adams et al., 1973). There is the substrate binding site, normally occupied by pyruvate or lactate, and an anion site of unknown function at the subunit interfaces. The data substantiate the occupation of the substrate binding site by competitive inhibitors such as oxamate.

The activity of lactate dehydrogenase is strongly influenced by physiological concentrations of chloride. Winer & Schwert (1958) observed that the K_m's for the enzyme are larger in Tris-HCl buffers than they are in phosphate buffers. Comparably, the dissociation of NADH[1] from lactate dehydrogenase also increases on the addition of chloride (Winer, 1963). These effects have been considered due to some mechanism other than direct competition for binding sites.

In studying the effects of halides on NADH binding by beef heart lactate dehydrogenase, I obtained evidence for the existence of two types of binding site having selective affinities for fluoride and chloride. This paper details some of the distinctive effects of these halides on the catalytic activity, NADH binding properties, and circular dichroism spectra of beef heart lactate dehydrogenase.

Materials and Methods

Reagents. NADH and sodium pyruvate were obtained from Sigma Chemical Co. All other chemicals were reagent grade. Solutions were prepared by using deionized glass-distilled water. The same buffer, 0.05 M potassium phosphate at pH 7.4, was used throughout the experiments. The pure H_4 isozyme was isolated chromatographically from a preparation of beef heart lactic dehydrogenase obtained from Worthington Biochemical Corp. (Pesce et al., 1964).

Catalytic Activity. All rate measurements were initial reaction velocities determined at 340 nm on a Cary 15 spectrophotometer. Concentrations of pyruvate and NADH in the range of their corresponding K_m's were examined. Cuvettes having 10-cm light paths were used when the NADH concentration was 10^{-5} M or less. A 1-cm light path was otherwise used.

Fluorescence Titrations. All measurements were carried out with the Hitachi Perkin-Elmer MPF-2A fluorometer. The excitation and emission wavelengths were fixed at 330 and 430 nm, respectively. The procedure for the manual titration of the enzyme with NADH was described by Anderson & Weber (1965).

Circular Dichroism. The circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism spectrophotometer. The base line contribution of the protein was eliminated by subtraction.

Results

Inhibition of Catalytic Activity by Fluoride. Kinetic experiments were carried out at varied NADH and pyruvate concentrations in order to detect inhibition of the catalytic activity of lactate dehydrogenase by fluoride. The results in Figure 1 show that fluoride is a competitive inhibitor with respect to pyruvate and a noncompetitive inhibitor with respect to NADH. Application of the equations derived by Novoa et al. (1959) to the data permits calculation of the values of K_I' and K_I'', the equilibrium constants for the dissociation of fluoride from the ternary enzyme-NADH-fluoride complex and the binary enzyme-fluoride complex, respectively. Since the NADH concentration used in Figure 1A is nearly saturating (its dissociation constant is near 6×10^{-7} M), the change in the slope of the Lineweaver-Burk plot with fluoride concentration (I) is directly related to K_I'. That is

$$\text{slope}/\text{slope}_0 = 1 + I/K_I' \qquad (1)$$

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[1] Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide; $\bar{n}$, the average number of moles of NADH bound per mole (136 000 g) of lactate dehydrogenase; β_0 and β, relative fluorescence yields of free and bound NADH, respectively; $[E_t]$, total concentration of enzyme in moles per liter calculated by using a molecular weight of 136 000 g; j, Hill coefficient; K_d, dissociation constant of enzyme-NADH complex; K_{app}, concentration of unbound NADH when 50% of the NADH binding sites are saturated; K_I' and K_I'', equilibrium constants for dissociation of fluoride from ternary and binary complexes, respectively; Tris, tris(hydroxymethyl)aminomethane.

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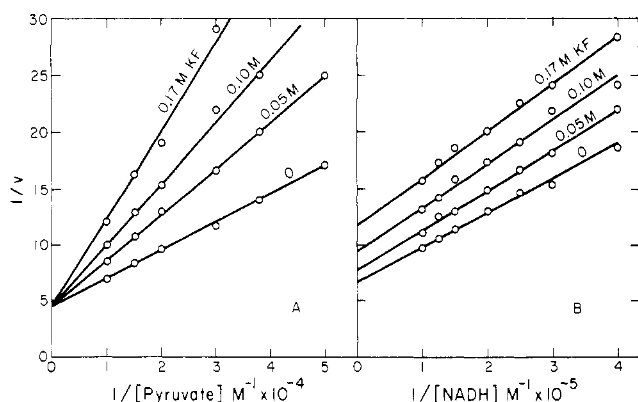


FIGURE 1: Lineweaver–Burk plots showing effect of KF on initial reaction rate (in arbitrary units) when (A) pyruvate concentration is varied and NADH concentration is constant (10^{-4} M) and (B) NADH concentration is varied and pyruvate concentration is constant (10^{-4} M). The measurements were carried out in 0.05 M potassium phosphate, pH 7.4, at 25 °C.

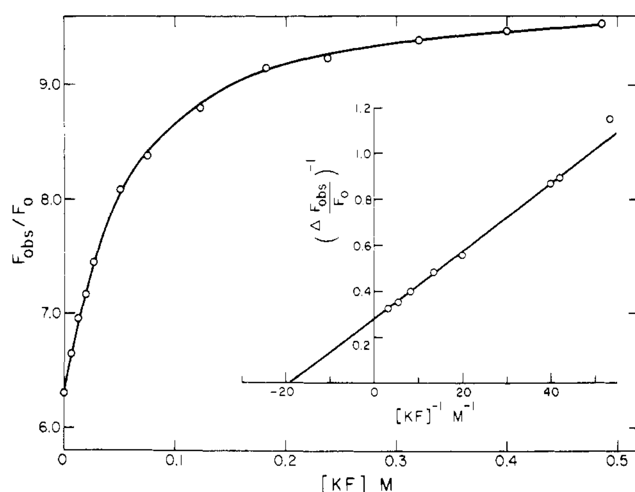


FIGURE 2: Effect of fluoride on fluorescence enhancement of bound NADH. Excitation, 330 nm; emission, 430 nm. Conditions: 2.0 mg/mL enzyme and 4.8×10^{-6} M NADH in 0.05 M potassium phosphate, pH 7.4, at 17 °C. The double-reciprocal plot of the *changes* in fluorescence enhancement with KF (inset) is used to calculate K_I'.

where slope$_0$ is the slope observed in the absence of fluoride. In the case of Figure 1B, the slopes are directly related to K_I''

$$\text{slope}/\text{slope}_0 = 1 + I/K_I'' \qquad (2)$$

Application of eq 1 to the data shows that the competitive inhibition with respect to pyruvate is described by $K_I'' = 0.08$ M. As expected from the nearly parallel lines of Figure 1B, K_I'' is larger than K_I'. $K_I'' = \sim 0.3$ M.

Ternary Complexes Containing Fluoride. The fluorescence quantum yield of the bound coenzyme usually increases when salts of dicarboxylic acids are added to solutions of lactate dehydrogenase and NADH (Winer & Schwert, 1959). Figure 2 shows that the fluorescence enhancement measured at 430 nm similarly increases when potassium fluoride is added. Since calculations using my value for K_d indicate that 99% of the NADH is initially bound, this change primarily reflects an increase in quantum yield. The double-reciprocal plot in the inset shows that the enhancement factor approaches 9.9 at infinite fluoride concentration and that K_I' is 0.053 M.

Fluoride appears to be part of a ternary complex with fluorescence properties similar to those of the ternary complex containing oxalate or a related anion (Winer & Schwert, 1959). A linked equilibrium of binary and ternary complexes is described by three *independent* dissociation constants. E represents the enzyme and R, the coenzyme

Table I: Anion Effects on NADH Binding by Beef Heart Lactate Dehydrogenase: Summary of Enhancement Factors, Dissociation Constants, and Hill Coefficients

salt	concn (M)	β/β_0	K_{app} (M)	j
KF	0.25	9.3	2.2×10^{-7}	0.89
KF	0.05	8.2	4.0×10^{-7}	0.90
no addition		6.3	6.1×10^{-7}	0.97
NaCl	0.1	6.9	1.6×10^{-6}	0.80
	0.1		1.6×10^{-6}	0.77
NaCl	0.12		1.7×10^{-6}	0.74
NaCl	0.15	7.05	2.0×10^{-6}	0.79
NaCl	0.18		2.4×10^{-6}	0.75
NaCl	0.4		3.8×10^{-6}	0.85
NaCl	0.5	7.05	4.4×10^{-6}	0.89
oxalate	0.05	48		

[a] All values were determined at 17 °C in 0.05 M potassium phosphate, pH 7.4.

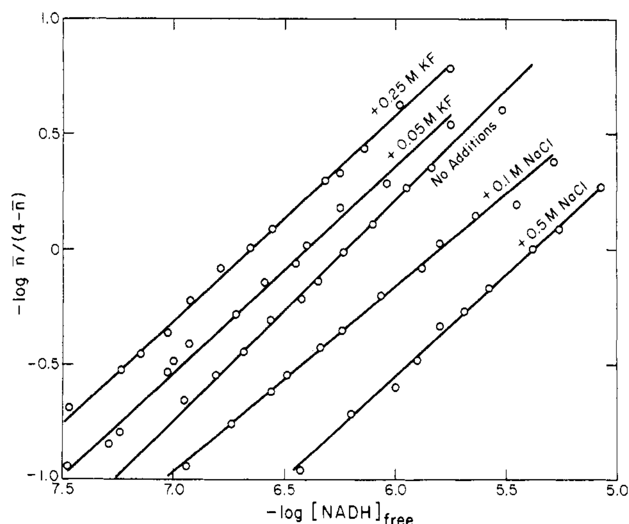
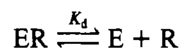
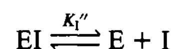


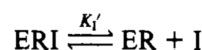
FIGURE 3: Hill plots of fluorescence titrations of beef heart lactate dehydrogenase with NADH. All the samples contained 0.05 M potassium phosphate, pH 7.4, plus the indicated additions of KF or NaCl. The temperature was 17 °C.



$$\mathrm{ER} \xrightleftharpoons{K_d} \mathrm{E} + \mathrm{R}$$



$$\mathrm{EI} \xrightleftharpoons{K_I''} \mathrm{E} + \mathrm{I}$$



$$\mathrm{ERI} \xrightleftharpoons{K_I'} \mathrm{ER} + \mathrm{I}$$

The use of the fluorescent-enhancement factor to calculate the fraction (f) of NADH bound gives

$$f = \frac{[\mathrm{ER}] + [\mathrm{ERI}]}{[\mathrm{R}] + [\mathrm{ER}] + [\mathrm{ERI}]}$$

[EI]/[E] and [ERI]/[ER] are constant ratios determined by the dissociation constants and by the fluoride concentration. The apparent dissociation constant (K_{app}) for NADH obtained by measuring its fluorescence enhancement is

$$K_{app} = \frac{(1 + I/K_I'')K_d}{(1 + I/K_I')}$$

The Hill plots in Figure 3 and the values of K_{app} in Table I show that the presence of fluoride augments NADH binding or that $K_I' < K_I''$. By substituting my values for K_d (6.1×10^{-7} M) and K_I' (0.053 M), I obtain values for K_I'' of 0.18 and 0.22 M from the results obtained with 0.05 and 0.25 M KF, respectively.

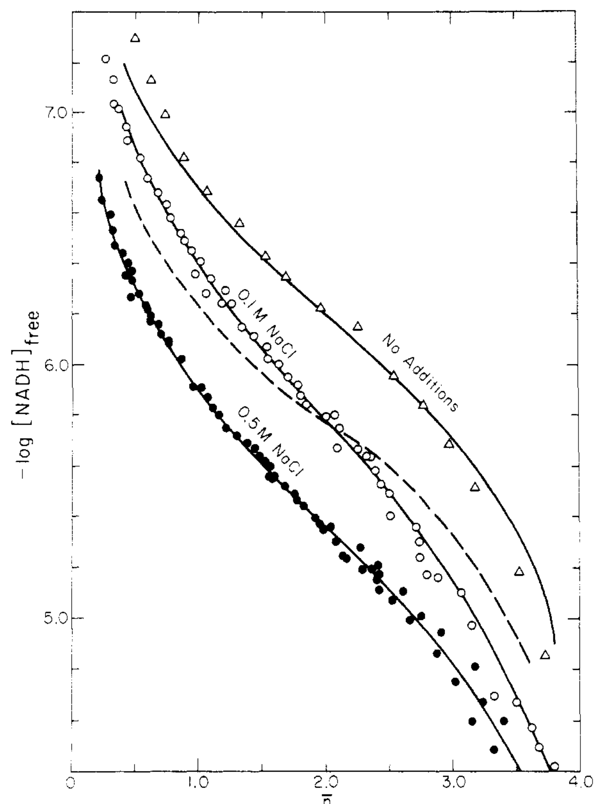


FIGURE 4: Effect of NaCl addition on bonding of NADH by beef heart lactate dehydrogenase. Data not included show that saturation with NADH is reached as $\bar{n}$ approaches four. Conditions are given under Figure 3. The dashed curve represents the span predicted for a Hill coefficient of 1.

Contrasting Effects of Chloride. To obtain additional information on the antagonistic effects of chloride on coenzyme binding by lactate dehydrogenase (Winer, 1963), I carried out fluorescence titrations spanning 90% or more of the entire saturation range of the NADH binding sites. I used the manual dilution method of Anderson & Weber (1965), which involves separate additions of constant proportions of NADH and enzyme to the solution in the cuvette. The enzyme concentration is varied to attain from 15 to 75% binding of the NADH. The ratio of NADH to enzyme is fixed at 2.5:1 for values of $\bar{n}$ between 1 and 1.5 and at 5:1 for values of $\bar{n}$ between 1 and 3.5. The solution is replaced after every five or six points to avoid the accumulation of error.

Three of these symmetric titration curves are shown in Figure 4, which illustrates the effects of 0.1 and 0.5 M NaCl on NADH binding by beef heart lactate dehydrogenase at pH 7.4. Figure 5 shows the large increase in K_{app}, the free NADH concentration when $\bar{n} = 2$, with increasing NaCl concentration. This trend, the opposite of that obtained with fluoride, may reflect mutually antagonistic binding of NADH and chloride ($K_I'' < K_I'$). Attempts to fit the values of K_{app} to the model for binary and ternary complexes gives values for K_I'' of 0.05 M and for K_I' of 0.6–1.0 M. In the presence of NaCl, the Hill coefficient for NADH binding undergoes a small but repeatable decline—reaching a minimum value of 0.75–0.8 at NaCl concentrations between 0.1 and 0.2 M (Figures 3 and 5). The replacement of NaCl with identical concentrations of KCl or CsCl gives the same results, showing that these effects are characteristic of the anion.

Independence of Protein Concentration. I performed the experiments described in the next paragraph to rule out reversible dissociation of the enzyme as a contributing factor to both the dramatic effect of NaCl on K_{app} and the subtle effect on the Hill coefficient. In the absence of protein–protein interactions, each free ligand concentration corresponds to a constant value of $\bar{n}$ which is independent of the total protein concentration ($[E_t]$). Dilution of a mixture of protein and ligand with a solution of free ligand matching its equilibrium concentration should produce no change in $\bar{n}$. The fluorescence of the solution is the sum of the fluorescence intensity of the free ligand ($\beta_0[NADH]_{free}$), which is constant when $\bar{n}$ is fixed, and the fluorescence intensity of the complex ($\bar{n}\beta[E_t]$) (Anderson & Weber, 1965)

$$F_{obsd} = \beta_0[NADH]_{free} + \beta\bar{n}[E_t] \quad (3)$$

A plot of F_{obsd} vs. $[E_t]$ is linear and allows calculation of β/β_0. Log–log plots are useful for demonstrating linearity over a large range of protein concentrations. To simplify, let $F_i = \beta_0[NADH]_{free}$

$$\log (F_{obsd} - F_i) = \log \beta + \log \bar{n} + \log [E_t]$$

Plots of $\log (F_{obsd} - F_i)$ vs. $\log [E_t]$ will have a slope of 1. Recall that

$$\frac{d \log (F_{obsd} - F_i)}{d \log [E_t]} = \frac{d(F_{obsd} - F_i)/(F_{obsd} - F_i)}{d[E_t]/[E_t]}$$

Since differentiation of eq 3 gives

$$\frac{d(F_{obsd} - F_i)/(F_{obsd} - F_i)}{d[E_t]/[E_t]} = 1 + \frac{d\bar{n}/\bar{n}}{d[E_t]/[E_t]}$$

deviations from unit slope directly reflect deviations in $\bar{n}$ with enzyme concentration.

The dilution experiment was carried out in 0.1 M NaCl and 0.05 M potassium phosphate (pH 7.4). Values of $\bar{n}$ equal to 1.0 and 3.06, corresponding to free NADH concentrations of 4.1×10^{-7} and 7.9×10^{-6} M, were chosen for dilution. Log–log plots of the results span a 20-fold range of enzyme concentration, from 0.1 to 2 μM when $\bar{n} = 1$ and from 0.15 to 3 μM when $\bar{n} = 3$. The line corresponding to $\bar{n} = 1$ has a slope of 0.95, and that corresponding to $\bar{n} = 3.06$ has a slope of 1.00. Thus, deviations in $\bar{n}$ with enzyme concentration are 5% or less when $\bar{n} = 1$, this being the approximate experimental error when $\bar{n}$ is near 1, and virtually 0 when $\bar{n} = 3$. The vertical displacement between the lines, 0.48 is the log of the ratio of the two values of $\bar{n}$. The value of β/β_0 determined from plots of F_{obsd} vs. $[E_t]$ is 6.8, in agreement with the value obtained directly under conditions of total (>99%) binding.

Circular Dichroism Spectra of Bound NADH. The circular dichroism corresponding to the 340-nm absorption band should be sensitive to changes in the immediate environment of the

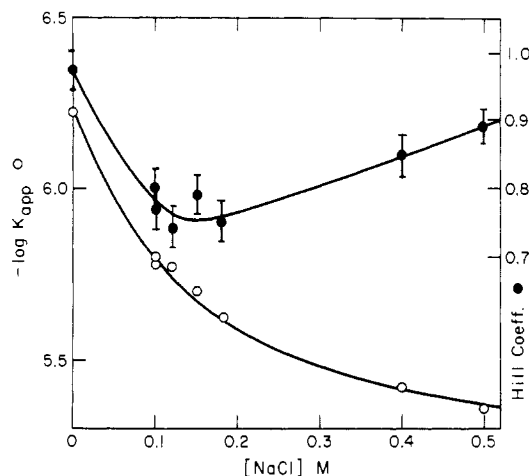


FIGURE 5: Effects of NaCl on K_{app}, free NADH concentration at $\bar{n} = 2$, and the Hill coefficient. Conditions are given under Figure 3.

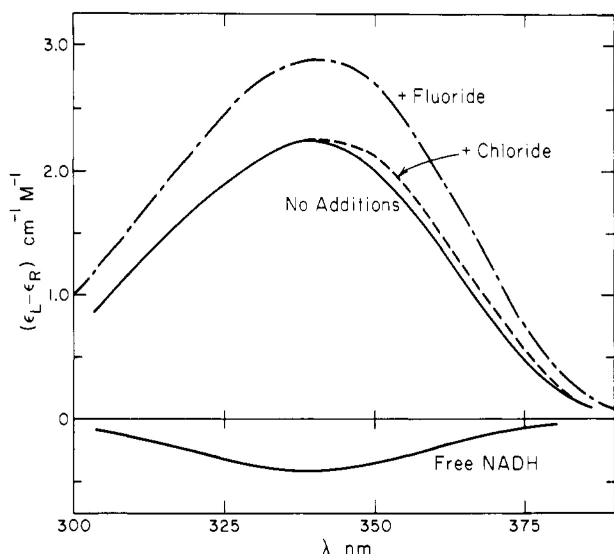


FIGURE 6: Circular dichroism spectra of bound NADH. The solutions contained 5 mg/mL lactate dehydrogenase, 7 × 10^{-5} M NADH, 0.05 M potassium phosphate (pH 7.4), and either 0.1 M NaCl or 0.5 M KF. The spectrum of free NADH is included for comparison.

bound NADH. The addition of either oxalate or fluoride produces large, distinctive changes in the circular dichroism spectrum of lactate dehydrogenase bound NADH. In contrast, the addition of chloride has negligible effects on the spectrum (Figure 6).

Discussion

Fluoride and chloride show contrasting behavior in their interaction with beef heart lactate dehydrogenase. The competitive inhibition of enzyme activity and the ternary complex detected in fluorescence and circular dichroism measurements indicate that fluoride occupies the pyruvate binding site. The interactions between fluoride and NADH are positive, with mutual enhancement of binding. The shift in the dissociation constant for fluoride, from ~0.2 M in the absence of NADH to 0.053 M in its presence, corresponds to an interaction energy (Weber, 1972) between fluoride and NADH of about −0.8 kcal/mol. Kolb & Weber (1975) determined an interaction energy of −1.3 kcal/mol for oxalate and NADH binding to chicken heart lactate dehydrogenase. The dissociation constants for fluoride determined fluorometrically are ~34% smaller than those obtained kinetically. By analogy to results obtained on oxamate binding to the enzyme (Winer & Schwert, 1959), this is probably due to the different temperatures used.

Most enzymes inhibited by fluoride are metalloproteins. However, lactate dehydrogenase does not contain bound metal ions (Vestling et al., 1963; Pesce et al., 1964). The X-ray studies show possible points of attachment for fluoride. Histidine-195, believed to hydrogen bond with the carbonyl of pyruvate or the hydroxyl of lactate (Holbrook et al., 1975), may possibly *hydrogen bond* to the *strongly electronegative* fluoride. Other possible interactions include ion pairing with arginine-109 or -171. This is the first report of fluoride inhibition of lactate dehydrogenase. Duplication of the results requires the use of adequately high fluoride concentrations together with nonsaturating pyruvate concentrations. In comparison to oxalate and oxamate (Winer & Schwert, 1959), fluoride is a weak inhibitor with K_I's more than 2 orders of magnitude larger.

The minimal effects of chloride on the fluorescence and circular dichroism of the bound NADH indicate that it binds primarily at site(s) other than the one occupied by fluoride. In contrast to the mutual enhancement of binding obtained with fluoride, the binding of chloride and NADH is strongly antagonistic, in fact, almost mutually exclusive or competitive. This is in agreement with Winer's report (Winer, 1963), which showed a similar dependence of NADH binding on the concentration of Tris-HCl. The Hill coefficient for NADH binding decreases slightly to 0.75–0.8 at NaCl concentrations between 0.1 and 0.2 M and increases to 0.9 or higher at the extremes of NaCl concentration.

There have been conflicting reports on the possible dissociation of beef heart lactate dehydrogenase. However, the binding of NADH by the enzyme in solutions containing 0.1 M NaCl is *independent* of protein concentration over the 20-fold range examined. Thus, neither the apparent anticooperativity or the overall sensitivity to chloride is linked to reversible dissociation of the enzyme. In fluorescence polarization experiments, I showed that the molecular weight of beef heart lactate dehydrogenase is constant throughout the concentration range of 2 × 10^{-8}–7 × 10^{-6} M, both in the presence and absence of NaCl (Anderson, 1969).

Adams et al. (1973) found that sulfate, citrate, and pyruvate can occupy either the subunit boundary or the active-center binding sites. The effects of fluoride and chloride are so well separated that the binding of the two anions probably takes place primarily at different types of sites, with fluoride bound at the active center and chloride bound at other unidentified sites. The binding of chloride to proteins may be commonplace. In deoxyhemoglobin, chloride is part of an interchain salt bridge between the C-terminal arginine of one α chain and the N-terminal valine of the other α chain. This salt bridge accounts for 20% of the alkaline Bohr effect in 0.1 M NaCl (Kilmartin et al., 1973; Rollema et al., 1975; O'Donnell et al., 1979).

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