Interaction of Chloride Ion with Horse Liver Alcohol Dehydrogenase–Reduced Nicotinamide Adenine Dinucleotide Complexes†

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ABSTRACT: The enhanced fluorescence emission of NADH when bound to horse liver alcohol dehydrogenase is diminished in the presence of chloride ion. Similarly, the extrinsic Cotton effect of bound NADH is removed when chloride is added to an enzyme–NADH complex. From these two observations it can be concluded that either chloride ion displaces coenzyme from the enzyme or binds simutaneously with coenzyme and alters extrinsic properties of bound enzyme. Three lines of evidence suggest that, indeed, the two ligands are bound simultaneously: (1) the polarization of fluorescence of NADH remains at 35–36% in the presence of chloride; thus the polarization of NADH is that of bound coenzyme even though the intensity of fluorescence emission is that of unbound NADH; (2) both the wavelengths for absorption and emission remain at that of enzyme-bound NADH; (3) the fluorescence emission of the enzyme's tryptophan, which is partially quenched in the presence of NADH, remains quenched in the presence of both NADH and chloride. We conclude that chloride can form ternary complexes with enzyme–NADH and the dissociation constant from this complex is between 0.2 and 1.0 M. The binding of chloride alters the enhanced fluorescence and other properties of bound NADH presumably through a conformational change in the enzyme.

Horse liver alcohol dehydrogenase (EC 1.1.1.1), a two-subunit enzyme, has not been generally considered a regulatory enzyme. Recent work from this laboratory (Iweibo and Weiner, 1972, 1973; Coleman *et al.*, 1972) as well as from others (Luisi and Favilla, 1970, 1972; Theorell and Tatemoto, 1971) now shows that the properties and reactivity of the enzyme are indeed modified by small molecules in solution. Both NAD^+ and NADH[1] were shown to bind not only to the two active sites of the enzyme, but to four additional sites (Weiner *et al.*, 1972; Iweibo and Weiner, 1973), as did an analog of NAD^+ (Weiner, 1969; Mildvan and Weiner, 1969). The occupancy of these ancillary sites inhibits the binding of substrates to the enzyme (Iweibo and Weiner, 1973).

There are many reports in the literature which conclude that chloride ion affects properties of horse liver alcohol dehydrogenase. Most significant are the ^{35}Cl nuclear magnetic resonance (nmr) studies of Ward and Happe (1971) and of Lindman *et al.* (1972) who found that chloride ion competes with NADH for enzyme. The optical rotation experiments of Li *et al.* (1963) led to an identical conclusion. NADH protects the enzyme against sulfhydryl inactivation by iodoacetate and chloride ion mimics this protective effect (Reynolds and McKinley-McKee, 1969). Thus, chloride ion is considered to exclude coenzyme.

Recent kinetic experiments revealed that the maximum velocity of the enzyme is increased threefold in the presence of 50 mM chloride ion (Shore and Gutfreund, 1970). Since the rate determining step for ethanol oxidation is the release of NADH from the enzyme–NADH complex, it is reasonable to suggest that these two ligands may be able to bind simultaneously to the enzyme as originally conjectured by Theorell *et al.* (1955).

The kinetic results which are in conflict with the binding studies suggest that there is more than one type of chloride ion binding site on the enzyme. Since we find that there is more than one type of coenzyme site (Iweibo and Weiner, 1973) and that the ^{35}Cl nuclear magnetic resonance and the optical rotation results require that the coenzyme and the anion compete for the same site, we have reinvestigated the interaction of chloride ion with alcohol dehydrogenase. This paper reports on the simultaneous binding of chloride and coenzyme to the enzyme and the accompanying paper (Coleman and Weiner, 1973) reports on the kinetic effects of chloride ion on the oxidation–reduction reactions.

Materials and Methods

Horse liver alcohol dehydrogenase was purchased from Worthington Biochemical Corp. and dialyzed against pH 7.0 sodium phosphate buffer, $\mu = 0.1$ at 4°, before use. All solutions were made in this buffer unless otherwise stated. NaCl was purchased from Mallinckrodt Chemical Works and NAD^+ and NADH from Boehringer-Mannheim. Isobutyramide was recrystallized from hot water. Tryptophan and *N*-acetyltryptophanamide were purchased from Sigma Chemical Co.

Alcohol dehydrogenase concentration was determined by absorbance at 280 nm using 0.455 the extinction of a 1-mg/ml solution (Cannon and McKay, 1969). The activity of the enzyme was assayed using the method of Dalziel (1957), and the concentration of active sites was determined by the isobutyramide titration method of Winer and Theorell (1960).

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer thermostated at 25°. Excitation and emission wavelengths were 280 and 350 nm, respectively, for tryptophan fluorescence, and 330 and 440

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[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

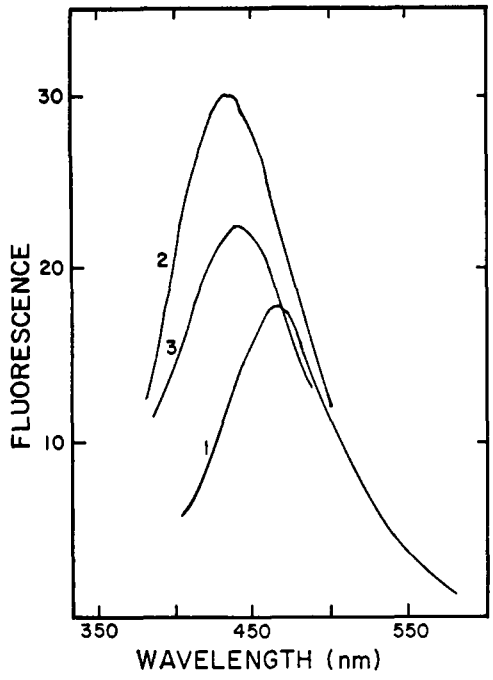

FIGURE 1: The fluorescence spectra of NADH, enzyme–NADH, and enzyme–NADH–chloride. Excitation was at 340 nm in pH 7.0 phosphate, μ = 0.1 at 25°, and the fluorescence was recorded on an Autograph x–y recorder. The spectra are: (1) 15.5 μM NADH; (2) 14.6 μM NADH and 4.4 μM horse liver alcohol dehydrogenase; (3) 13.7 μM NADH, 4.05 μM alcohol dehydrogenase, and 220 mM NaCl.

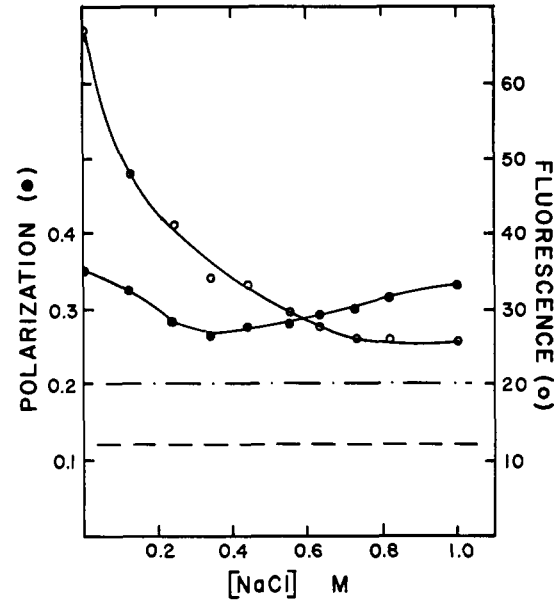

FIGURE 2: The titration of the enzyme–NADH complex by NaCl. The excitation and emission wavelengths were 340 and 440 nm, respectively. Both fluorescence (○) and fluorescence polarization (●) were measured in the same solution after each addition of NaCl. The line at a polarization value of 0.12 (- - -) represents the polarization of free NADH in the presence or absence of NaCl. The line at a fluorescence value of 0.20 (–·–) represents the value of fluorescence measured for the same concentration of free NADH in the presence or absence of NaCl. Conditions are as in Figure 1, curve 2.

nm, respectively, for coenzyme fluorescence. Glan prisms were used for the polarization of fluorescence experiments. Self-absorption effects on fluorescence were minimized by off-center displacement of the cuvet chamber as described by Iweibo and Weiner (1972). Spectra were recorded on an Autograph x–y recorder.

Absorbances were measured on a Gilford spectrophotometer. The extinction coefficients used for NAD^+ and NADH were 17.9×10^3 M^{-1} cm^{-1} (259 nm) and 6.22×10^3 M^{-1} cm^{-1} (340 nm), respectively.

Optical rotatory dispersion experiments were performed on a Cary 60 spectropolarimeter at 23° scanning from 550 to 230 nm. Calculations are described in Coleman *et al.* (1972), using the formula from Urnes and Doty (1961)

$$[m']_\lambda = \frac{3}{(n^2 + 2)} \frac{100\alpha MRW}{dc} \quad (1)$$

where n is the refractive index and $3/(n^2 + 2)$ was obtained from Weast (1969) and Sober (1968) for all NaCl concentrations; MRW, the mean residue weight, was taken as 104.8 from Theorell *et al.* (1966); d is the pathlength in decimeters, 0.1 dm; c is the protein concentration in g/100 ml; $[m']_\lambda$ is the mean residue rotation at wavelength λ.

Results

Absorbance Properties of Alcohol Dehydrogenase in the Presence and Absence of Chloride Ion. The visible absorbance spectrum of NADH is altered when the coenzyme binds to horse liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951). The optimum absorbance of the unbound coenzyme is at 340 nm, and the addition of enzyme shifts this to 325 nm. No change in the absorbance spectrum of unbound NADH was observed when NaCl was added in concentrations up to 0.5 M. It was also observed that there was no effect of up to 1.0 M chloride ion upon the absorbance of horse liver alcohol dehydrogenase when the anion was added to the free enzyme, or to enzyme–NADH and enzyme–NADH–isobutyramide complexes.

Fluorescence Intensity of Alcohol Dehydrogenase in the Presence and Absence of Chloride Ion. Coenzymes and substrates, when bound to alcohol dehydrogenase, quench the tryptophan fluorescence (Luisi and Favilla, 1970; Theorell and Tatemoto, 1971; Iweibo and Weiner, 1972). The addition of up to 1.0 M NaCl caused no change in the fluorescence emission of the enzyme, nor did chloride ion cause tryptophan fluorescence changes in the enzyme complexes with NAD^+, NADH, or NADH–isobutyramide.

Upon binding to horse liver alcohol dehydrogenase, the fluorescence of NADH undergoes a blue shift and a marked enhancement (Boyer and Theorell, 1956). When chloride ion, with either sodium, potassium, or lithium as the counterion, is titrated into a preformed binary complex of enzyme–NADH the enhanced fluorescence of bound coenzyme is quenched (Figure 1). The final fluorescence is 10–20% greater than expected for the same concentration of unbound NADH (Figure 2), and the resulting spectrum (Figure 1) has an optimal fluorescence at 440 nm rather than at 470 nm. The shorter wavelength is that observed for enzyme-bound NADH, the longer for free NADH. The decrease in fluorescence allows the calculation of a dissociation constant for chloride from the enzyme. A double reciprocal plot of the data in Figure 2 revealed that K_D = 0.2 M.

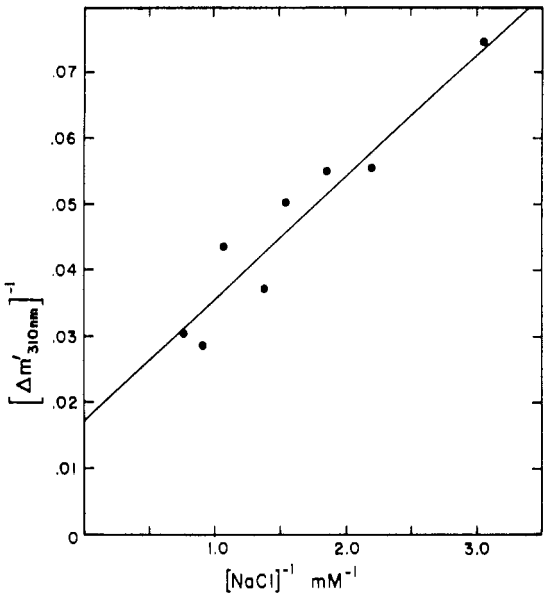

FIGURE 3: A Klotz plot for the loss of optical rotation of the extrinsic Cotton effect of bound NADH in the presence of increasing concentrations of chloride. The mean residue rotation [m'] at 310 nm in pH 7.0 phosphate, μ = 0.1 at 25°, was calculated according to eq 1. The data were treated according to the method of Klotz (1953), and a K_D of 1.1 ± 0.3 M was obtained.

There was no effect of chloride ion on the fluorescence of free NADH nor on the fluorescence of tryptophan or *N*-acetyltryptophanamide. Sodium nitrate or phosphate at the same ionic strength did not quench the fluorescence of bound NADH as well as did sodium chloride. Thus, the effect is not due to ionic strength but to anions interacting with the enzyme.

Polarization of Fluorescence of NADH in the Presence and Absence of Chloride. NADH bound to horse liver alcohol dehydrogenase has a polarization of 0.36, and that of unbound NADH is 0.12 (Velick, 1961). The polarization of NADH fluorescence at either 440 or 470 nm was observed as a function of chloride ion concentration in the presence of the preformed enzyme–NADH binary complex (Figure 2). The polarization remains near 0.35, even in the presence of above 1.0 M chloride ion. This and the lack of a shift of the fluorescence maximum suggest that the chloride ion did not displace NADH from the enzyme, but only quenched the emission.

Optical Rotatory Dispersion of Alcohol Dehydrogenase in the Presence and Absence of Chloride. NADH bound to horse liver alcohol dehydrogenase has an extrinsic Cotton effect (Li *et al.*, 1963; Rosenberg *et al.*, 1965). The rotation at 310 nm due to enzyme–NADH complex is lost upon addition of chloride ion (Li *et al.*, 1963). A dissociation constant of 1.1 M was calculated from the change in optical rotation at 310 nm (Figure 3).

Discussion

Recent reports from many laboratories have shown that properties of horse liver alcohol dehydrogenase are capable of being regulated (Theorell and Tatemoto, 1971; Luisi and Favilla, 1970, 1972; Iweibo and Weiner, 1972). Iweibo and Weiner (1973) have reported modification of *in vitro* activity caused by the binding of coenzyme molecules to regulatory sites. The main effect of high chloride ion concentrations on alcohol dehydrogenase had been interpreted as a release of coenzyme from the enzyme. We have now further investigated the phenomena associated with chloride ion, coenzyme, and horse liver alcohol dehydrogenase in order to determine how anions affect the enzyme's interactions with coenzyme.

Tryptophan fluorescence of alcohol dehydrogenase is quenched when coenzyme and/or substrate is bound to the enzyme. Whereas the addition of chloride to a free enzyme solution causes no such change, its addition also does not increase the already quenched aromatic amino acid fluorescence of an enzyme–binary coenzyme. The latter experiments suggest that it does not cause the release of coenzyme from the enzyme.

The addition of chloride ion to a preformed enzyme–NADH complex reduces the fluorescence of bound NADH. The observation of the quenching of fluorescence is consistent with the previous reports cited above wherein chloride ion and NADH are thought to be mutually competitive. However, the spectrum of the quenched coenzyme fluorescence (emission at 435 nm) is suggestive of an enzyme–NADH complex rather than of free NADH (emission at 470 nm). Furthermore, direct evidence that the enzyme–NADH binary complex exists in the presence of chloride was obtained from the polarization of NADH fluorescence experiments. Even at high anion concentrations, the polarization remained at about 0.35 compared with the value of 0.12 for free coenzyme. These experiments indicate that coenzyme is still bound to the protein simultaneously with chloride ion.

In the accompanying paper (Coleman and Weiner, 1973) evidence for at least two different types of chloride ion binding sites is presented. It may be that the small changes in NADH polarization of fluorescence (Figure 2) are related to the binding of chloride ion at different sites. In fact, one NADH molecule may be initially removed from the two active sites, as suggested by Lindman *et al.*, (1972), or more loosely held, when only the first chloride binding sites are occupied. When the second type of chloride ion site begins to fill, the coenzyme may assume a structure which possesses a higher degree of polarization. Thus, the conformation of the enzyme may be a function of chloride ion concentration. Evidence for conformational change in horse liver alcohol dehydrogenase upon ligand binding is found in other physical studies (Rosenberg *et al.*, 1965; Bränden *et al.*, 1970; and Iweibo and Weiner, 1973). Thus, the decrease in the fluorescence intensity as well as the loss of the extrinsic Cotton effect caused by addition of chloride ion is interpreted as the result of a conformational change in the enzyme, and not a loss of bound coenzyme.

The conclusion based on the fluorescence experiments is further corroborated by the coenzyme absorbance experiments. Coincident with the binding of coenzyme to horse liver alcohol dehydrogenase is a blue shift from 340 to 325 nm in the absorbance spectrum. There is no change in the coenzyme absorbance of the enzyme–NADH complex upon the addition of up to 1.0 M NaCl or KCl.

We conclude, therefore, that chloride ion can bind to horse liver alcohol dehydrogenase concurrently with coenzyme, that there may be more than one type of site for chloride on the enzyme, and that the binding of chloride can cause a change in the conformation of the protein. This change may be similar in effect to the changes caused by the binding of coenzyme to the regulatory binding sites for coenzyme.

To further elucidate the binding of chloride, kinetics and additional binding experiments were performed and are reported in the accompanying paper (Coleman and Weiner, 1973).

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Simultaneous Binding of Competitive Ligands to Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: Chloride ion binds to at least two different types of sites on horse liver alcohol dehydrogenase. In initial velocity experiments with coenzyme as the varied component at saturating alcohol or aldehyde levels, chloride was found to be competitive toward coenzyme with a K_I of 30–60 mM. Similarly, chloride was a competitive inhibitor against ethanol at saturating oxidized nicotinamide adenine dinucleotide (NAD^+) levels (K_I = 100 mM). However, chloride was a noncompetitive inhibitor against either acetaldehyde reduction or isobutyramide binding at saturating NADH levels. The inhibition of acetaldehyde is mainly due to chloride binding to the enzyme–NAD^+ complex formed from the reduction of aldehyde, thus inhibiting the release of NAD^+ from the enzyme. Some enzyme–NADH–aldehyde–Cl complex can also form which accounts for the inhibition being noncompetitive rather than uncompetitive. Chloride appears to bind to free enzyme excluding coenzyme and also simultaneously with coenzyme. Thus, there have to be at least two different types of chloride binding sites. The fact that the anion is competitive toward ethanol but binds simultaneously with isobutyramide (a specific aldehyde inhibitor) suggests that there may even be additional specific anion binding sites on the enzyme.

Bromide ion (Plane and Theorell, 1961) and chloride ion (Theorell *et al.*, 1955) are competitive inhibitors for NADH[1] and NAD^+ with horse liver alcohol dehydrogenase (EC 1.1.1.1). Since we have found by static methods that chloride can bind to enzyme concurrently with NADH (Coleman and Weiner, 1973), we have now performed kinetic experiments in the presence and absence of chloride ion in order to resolve

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[1] Abbreviations used are: enzyme, one subunit of alcohol dehydrogenase; K_D, dissociation constant; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.