Binding of Coenzyme, Coenzyme Fragments, and Inhibitors to Native and Carboxymethylated Horse Liver Alcohol Dehydrogenase from Chlorine-35 Nuclear Magnetic Resonance Quadrupole Relaxation†

Inger Andersson, Michael Zeppezauer, Thomas Bull,* Roland Einarsson,‡ Jan-Erik Norne, and Bjorn Lindman

ABSTRACT: ^{35}Cl nuclear magnetic resonance (NMR) quadrupole relaxation measurements have been performed in order to get a detailed picture of the interaction of coenzyme fragments and coenzyme competitive inhibitors with anion binding sites within the active site region of native and carboxymethylated horse liver alcohol dehydrogenase (LADH). A marked complexity of the anion binding was demonstrated with the existence of at least two anion binding sites within the coenzyme binding domain. Both the magnitude of the ^{35}Cl relaxation rate and its change with added ligand point to anion binding to positively charged amino acid residues and in no case could any anion binding to the Zn atoms be demonstrated. A correlation of the present data with X-ray crystallographic data suggests Arg-47 and Arg-271 to be involved in anion binding. The sequences of increasing affinity among the ligands investigated (inter alia adenosine, adenosine monophosphate, adenosine diphosphate, adenosinediphosphoribose, salicylate, 8-anilino-1-naphthalenesulfonate, NADH) are quite different for the two sites and it was also found that the sites are not independent but show an internal cooperativity. By studying the ^{35}Cl NMR quadrupole relaxation in solutions of LADH in the presence of 1,10-phenanthroline and either NADH or $Pt(CN)_4^{2-}$, it was found that the coenzyme molecule is sterically hindered to bind to the enzyme in the presence of the chelator; this applies not, however, for $Pt(CN)_4^{2-}$. Carboxymethylation of Cys-46 liganded to the catalytic zinc has no or little effect on the anion-binding properties of LADH; the modification does not seem to alter the zinc into a strong anion-binding site. By studies of both the longitudinal (T_1) and transverse (T_2) relaxation times of ^{35}Cl as a function of added coenzyme or another ligand, it could be established that the anion-binding group has a marked and rapid internal mobility.

Structural studies have revealed great similarities in the coenzyme binding domains of NAD-dependent dehydrogenases such as lactate dehydrogenase (LDH), malate dehydrogenase, glyceraldehyde-phosphate dehydrogenase, and horse liver alcohol dehydrogenase (LADH), whereas the catalytic domains of these enzymes show structural individualities (Rossman et al., 1975). The obligatory pathway of catalysis in most of these cases seems to be the binding of the coenzyme molecule which triggers a conformational change of the protein. This is then followed by a substrate activation which makes oxidoreduction possible (Adams et al., 1973). While in LDH, adenosine plays the role of trigger of this conformational change, the whole coenzyme molecule is required in the case of LADH. The substrate activation is not only induced by the amino acid groups of the protein but the metal ion has also an important function. Thus, the detailed picture of the mechanism of the binding of the coenzyme, yet not fully understood, is important for a complete understanding of catalysis.

In this investigation, we want to give a contribution on a physical basis to, in particular, the charge interactions important in the process of coenzyme binding. The interaction of coenzyme and protein is complex; from structural studies as well as kinetic/thermodynamic investigations in solution, it has been possible to distinguish between the following types of interactions (Brändén et al., 1975).

(i) Hydrophobic interactions occur between the adenine ring and the corresponding adenine binding pocket at one end of the coenzyme binding crevice. The binding site of the nicotinamide ring is built up of predominantly nonpolar residues; thus, the binding of the nicotinamide ring in NADH is also governed by hydrophobic interactions.

(ii) Hydrogen bonding is critical for the alignment of both ribose moieties. There are hydrogen bonds between the carboxyl group of Asp-223 and the *O*-2′-hydroxy group of the adenine ribose and between the main chain carbonyl oxygens of Ile-269 and Gly-293, and O-3′ and O-2′ of the nicotinamide ribose.

(iii) Electrostatic interactions are important for the interactions between the pyrophosphate bridge and Arg-47 and (in part) between the nicotinamide ring of NAD^+ and the zinc-bound hydroxide ion.

The differentiation between these various kinds of interactions has shown to be by no means an easy task, since the binding of the whole coenzyme certainly is a concerted process involving all kinds of forces. Earlier work toward this end has given the following picture. The interactions between the adenosinediphosphoribose moiety and the protein determine to a large extent the binding of NADH which was inferred from the similar pH dependence of the binding equilibria of ADPR[1] and NADH (Theorell & Yonetani, 1963; Yonetani, 1963). In NADH, the nicotinamide ring provides for the higher affinity as compared with ADPR by its hydrophobic interaction which is pH independent. Later Gunnarsson et

† From Fachbereich 15, Fachrichtung Biochemie, Universität des Saarlandes, D-66 Saarbrücken 11, West Germany (I.A. and M.Z.), and the Divisions of Physical Chemistry 1 (B.L.) and 2 (T.B. and J.N.), Chemical Center, S-220 07 Lund, Sweden. *Received December 5, 1978.* This work was supported by grants from the Swedish Natural Science Research Council and Deutsche Forschungsgemeinschaft Fonds der Chem. Industrie.

* Present address: Office of Technology Assessment, U.S. Congress, Washington, D.C.

‡ Present address: Pharmacia AB, Uppsala, Sweden.

[1] Abbreviation used: ADPR, adenosinediphosphoribose.

0006-2960/79/0418-3407$01.00/0

al. (1974) found that the pH dependence of the binding of adenosine monophosphate (AMP) parallels that of ADPR. Similar results were obtained by Reynolds et al. (1970) by studying the inhibitory effect of adenosine and AMP at various pH and ionic strengths. They concluded that the phosphate group of AMP has a strong influence on binding, probably due to interaction with a positive group on the enzyme. An example of a rather localized effect is the probable ionization of the zinc-bound water ($H_2O \rightarrow OH^-$), which strengthens the interaction between the positively charged nicotinium ring and the metal in the binding of NAD^+ and, thus, accounts for the different pH dependence of the binding of this particular ligand (Brändén et al., 1975).

Recent crystallographic studies of the binding of the coenzyme analogue ADPR have revealed stereochemical details of the interaction of the major part of the coenzyme molecule with the protein structure. From model building studies and recent X-ray investigations, it is inferred that the nicotinamide moiety of the coenzyme indeed is bound in close vicinity to the catalytic metal ion (Eklund et al., 1976). Special aspects of the interaction of the anion binding site with large anions such as $Pt(CN_4)^{2-}$ have been revealed in a structural study on native and carboxymethylated horse liver alcohol dehydrogenase (Zeppezauer et al., 1975). For a detailed understanding of the process of coenzyme binding, it will be necessary to develop methods which provide correlations between structural and other types of investigations and which give information on localized events within the coenzyme binding domain. Electrostatic interactions have, for example, been studied by means of kinetic studies (Gunnarsson et al., 1974) and halogen NMR quadrupole relaxation (Norne et al., 1975b) by using negatively charged metal complexes as probes. These studies clearly indicate the participation of Arg-47 in the binding of the coenzyme and furthermore indicate cooperativity in the binding process and the existence of more than one anion binding site.

In concern of the role of the metal ions in liver alcohol dehydrogenase, it has been questioned whether the catalytic metal is involved in anion binding or not. NMR quadrupole relaxation can be used to differentiate between anion binding to a metal ion and general anion binding (Norne et al., 1975b; Lindman & Forsén, 1976; Bull et al., 1978) On comparison of quadrupole coupling constants, some metal-containing proteins, among them horse liver alcohol dehydrogenase, fall into the same class as most of the non-metal-containing proteins studied. Moreover, addition of 1,10-phenanthroline, which binds to the active site zinc in LADH, has no effect on the ^{35}Cl relaxation of a solution containing LADH and Cl^- (Zeppezauer et al., 1969; Ward & Happe, 1971). This indicates that 1,10-phenanthroline does not interfere directly with an anion-binding site. However, by introducing a chemical modification on one of the ligands of the catalytic zinc in alcohol dehydrogenase (Zeppezauer et al., 1975), it should be possible to change the charge on the metal so that it might be able to bind anions directly. A method for selective carboxymethylation of Cys-46 in horse liver alcohol dehydrogenase has been described earlier (Zeppezauer et al., 1975).

The aim of this ^{35}Cl NMR quadrupole relaxation study was to give a more detailed picture of the coenzyme–protein interaction by studying the mutual interaction of coenzyme fragments and coenzyme competitive inhibitors with all possible anion binding sites in the active site of native and carboxymethylated horse liver alcohol dehydrogenase. The quadrupole relaxation method for studying ion binding to macromolecules is based on a change in the ion's NMR relaxation rate by orders of magnitude on binding to a macromolecule. Both a brief sketchy review (Forsén & Lindman, 1978) and a detailed account (Lindman & Forsén, 1976) have recently been presented.

Experimental Procedure

Materials. Horse liver alcohol dehydrogenase (LADH) (lots no. 12 86 442 and 10 37 243) was obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). Prior to use, the enzyme crystal suspension was centrifuged; the crystals were dissolved in a small volume of 0.05 M Tris-HCl buffer at pH 8.4 and containing 0.2 M KCl. After centrifugation, the enzyme was recrystallized by means of equilibrium dialysis against Tris buffer containing 20% (v/v) of *tert*-butyl alcohol. Stock solutions were then prepared as mentioned above and care was taken to remove all alcohol by repeated dialysis against buffer.

The enzyme was assayed according to the method of Dalziel (1957); the specific activity was determined to lie between 14.0 and 15.0 mg^{-1} min^{-1} (ΔA_{340}) for all samples of native enzyme in this investigation. NAD^+ titration in the presence of pyrazole was performed according to the method of Einarsson et al. (1976); active site content was found to vary between 90 and 94%. Protein concentration was determined by the measurement of absorption at 280 nm by using an absorption coefficient of 0.455 mg^{-1} cm^2.

NAD^+ and NADH were obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). The purity of the oxidized coenzyme was determined enzymatically and the concentration of the reduced coenzyme was determined spectrophotometrically by using an absorption coefficient of 6.22 mM^{-1} cm^{-1}.

8-Anilino-1-naphthalenesulfonic acid was bought from E. Merck (Darmstadt, F.R.G.) and was recrystallized in hot water. Imidazole was obtained from E. Merck (Darmstadt, F.R.G.) and was recrystallized twice from toluene. Iodoacetic acid was purchased from Sigma Chemical Co. and recrystallized twice from heptane. All other chemicals were commercial samples of highest available purity and used without further purification. Adenosine, AMP, ADP, and ADPR were bought from Boehringer Mannheim GmbH (Mannheim, F.R.G.), potassium tetracyanoplatinate(II) was from Degussa (Frankfurt, F.R.G.), and salicylic acid was from BDH Chemicals (Poole).

Buffer solutions were prepared from quartz double-distilled water and Ultrapure Tris (tris(hydroxymethyl)aminomethane) (Schwarz/Mann, Orangeburg, NY) and from Ultrapure KCl and HCl (E. Merck, Darmstadt, F.R.G.).

Methods. Horse liver alcohol dehydrogenase was specifically carboxymethylated at Cys-46 according to the method of Zeppezauer et al. (1975).

^{35}Cl NMR studies were performed at 9.80 MHz by using a modified Varian XL-100-15-spectrometer operating in the Fourier transform mode. The sample temperature was maintained at 10 (±0.5 °C) by a stream of dry thermostated nitrogen gas. The excess transverse relaxation rate, $R_{2,ex}$, was calculated from the line width at half-height of the NMR absorption curve according to $1/T_{2,ex} = R_{2,ex} = \pi(\Delta\nu_{obsd} - \Delta\nu_0)$, where $\Delta\nu_{obsd}$ is the observed line width and $\Delta\nu_0$ that obtained in the absence of protein. Relaxation time measurements were performed on a Bruker Bkr-322 s spectrometer by using the π–t–$\pi/2$ pulse sequence for the longitudinal relaxation time (T_1) and the Meiboom-Gill-Carr-Purcell sequence for the transverse relaxation time (T_2). The standard deviation of the fits of the magnetization was ca. 5% or less in the determi-

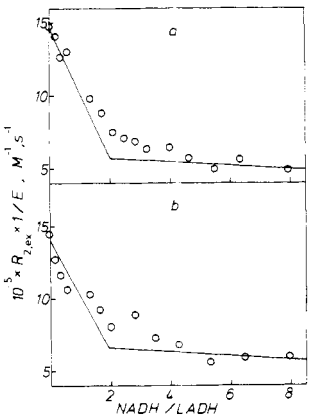

FIGURE 1: Transverse excess ^{35}Cl relaxation rate $1/T_{2,ex} = R_{2,ex}$ divided by the enzyme concentration, E, as a function of the molar ratio of the reduced coenzyme (NADH) to horse liver alcohol dehydrogenase (LADH). (a) Native horse liver alcohol dehydrogenase, 82.7 μM; (b) carboxymethylated horse liver alcohol dehydrogenase, 87.5 μM. The experiments were carried out at 10 °C and the Cl^- concentration was 0.5 M.

nations of T_1 and T_2 and the estimated error in the line-width measurements was ca. 5%.

Results

Titration of Native and Carboxymethylated Horse Liver Alcohol Dehydrogenase with Reduced Coenzyme, NADH. As earlier reported, solutions of horse liver alcohol dehydrogenase and chloride ions show an enhanced ^{35}Cl NMR relaxation compared with that of chloride ions alone (Norne et al., 1973; Lindman et al., 1972). On addition of ligands competing with chloride for the binding sites, the relaxation decreases due to the competition. If the ligand competes directly with Cl^-, it is possible to draw conclusions on the relative affinities of the ligands from the shape of the titration curve. A ligand binding much more strongly than Cl^- gives a stoichiometric release of Cl^- ions and, therefore, the ^{35}Cl relaxation rate at first decreases and then assumes an essentially constant value. Such a behavior was observed on adding reduced coenzyme, NADH, to a solution of horse liver alcohol dehydrogenase and Cl^- ions. Two molecules of NADH per dimeric enzyme molecule reduce the ^{35}Cl line width considerably, while further addition has only a small effect (see also Ward & Happe, 1971). Thus, it was possible to estimate the stoichiometry of the interaction; this is illustrated in Figure 1, which also shows the same titration curve with carboxymethylated enzyme. As can be seen, the two curves are almost identical, except that an overall somewhat lesser decrease in relaxation rate upon addition of NADH is observed in the case of the carboxymethylated enzyme. The same type of titration curves was obtained in studies of T_1, the longitudinal relaxation time, as an indicator; this is shown in Figure 2. The same stoichiometry is observed in this case. By comparing the effect of NADH addition on T_1 and T_2, it is possible to gain insight into inter alia internal motion phenomena; this aspect is considered below.

In the presence of ethanol, the NADH titration curve displays a considerable curvature pointing to a reduced affinity for NADH binding. However, this effect has been shown difficult to document in detail in a reproducible way.

Competition Experiments with Coenzyme Fragments and Coenzyme Competitive Inhibitors. It was considered of interest to map in detail the loci of anion binding in the coenzyme binding region and the factors influencing it. To achieve this, we have studied the competition between Cl^- and fragments of the coenzyme NADH as well as some coenzyme competitive inhibitors. The binding positions of these species have been

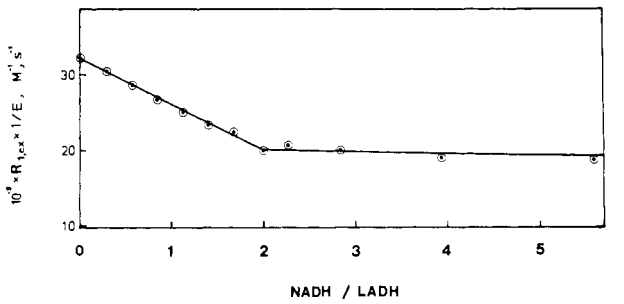

FIGURE 2: Longitudinal excess ^{35}Cl relaxation rate $1/T_{1,ex} = R_{1,ex}$ divided by the enzyme concentration, E, as a function of the molar ratio of reduced coenzyme (NADH) to horse liver alcohol dehydrogenase (LADH). The solutions contained 0.13 mM LADH and 0.5 M KCl. The pH was 8.4 and the temperature 6 °C.

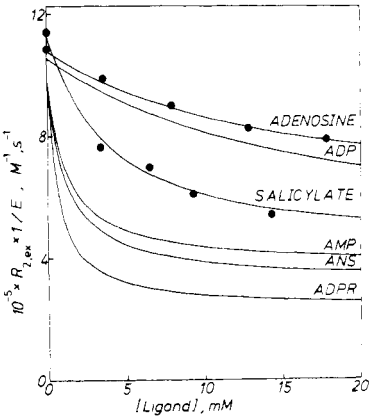

FIGURE 3: Transverse excess ^{35}Cl relaxation $1/T_{2,ex} = R_{2,ex}$ divided by the enzyme concentration, E, for the titration of an LADH–Cl^- solution with adenosine, adenosine monophosphate, adenosine diphosphate, adenosinediphosphoribose, salicylate, and 8-anilino-1-naphthalenesulfonate. The Cl^- concentration was 0.5 M and the LADH concentration in the range 62.8–91.7 μM. For the sake of clearness, experimental points are indicated just for a couple of the curves; the others, however, show the same pattern.

determined by X-ray crystallographic investigations (Eklund et al., 1976; Einarsson et al., 1974). In Figure 3 is given the ^{35}Cl excess transverse relaxation rate as a function of the concentration of added adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine diphosphoribose (ADPR), salicylate, and 8-anilino-1-naphthalenesulfonate (Ans). All these ligands bind with moderate affinity, and in this case the relaxation rate is given by eq 1. Here C_p, and C_{Cl}, and C_L are the total concentrations

$$R_{ex} = C_p \sum \frac{n_i K_{iCl} R_{bi}}{1 + K_{iCl} C_{Cl} + K_{iL} C_L} \qquad (1)$$

of protein, chloride, and added ligand, respectively, n_i is the number of equivalent sites of type i, characterized by the intrinsic binding constants K_{iCl} and K_{iL} and the relaxation rate R_{bi}.

It can be inferred from Figure 3 that all the ligands represented compete with Cl^- for certain sites on alcohol dehydrogenase but that the titration curves differ greatly. In order to make a comparison between different ligands feasible, we have analyzed the data on the basis of eq 1, assuming that there is one type of binding site for the added ligand. Equation 1 may then be rewritten:

$$\frac{R_{ex}}{C_p} = \frac{nK_{Cl}R_b}{1 + K_{Cl}C_{Cl} + K_L C_L} + A \qquad (2)$$

A is a residual relaxation rate (per unit protein concentration)

Table I: Ligand Binding to LADH[a]

	K_D (μM)	K_L/K_{Cl}	$A \times 10^{-5}$ (s^{-1} M^{-1})
adenosine	17200[b]	0.064	5.2
adenosine monophosphate	32[b]	0.775	3.7
adenosine diphosphate	180[b]	0.055	*f*
adenosine diphosphoribose	10.5[b] 35[c]	1.56	2.3
salicylate	1140[d]	0.284	4.1
8-anilino-1-naphthalene-sulfonate	74[e]	0.764	3.1

[a] K_D is the ligand dissociation constant in phosphate buffer taken from the literature. K_L/K_{Cl} is the binding constant relative to that of Cl^- as deduced from ^{35}Cl relaxation studies. A is the residual ^{35}Cl relaxation divided by the enzyme concentration when the effect of the ligand is saturated. [b] Reynolds et al. (1970). [c] Li & Vallee (1964). [d] Einarsson et al. (1974). [e] Einarsson (1974). [f] Uncertain but almost as high as for adenosine.

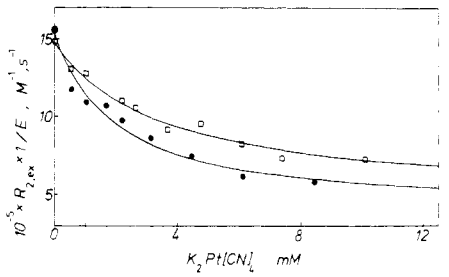

FIGURE 4: Transverse excess ^{35}Cl relaxation rate $1/T_{2,ex} = R_{2,ex}$ divided by the enzyme concentration, E, as a function of added $K_2Pt(CN)_4^{2-}$ for aqueous protein solutions containing 0.5 M KCl at 10 °C. Native horse liver alcohol dehydrogenase (●), 82.7 μM (0.53; 3.91×10^{-5}); (□) carboxymethylated horse liver alcohol dehydrogenase, 87.5 μM (0.283; 4.53×10^{-5}). The curves in the figure are calculated from the relative binding constants K_L/K_{Cl}. This value as well as the A values are given in the parentheses.

when the effect of the added ligand is saturated. It is clear from the NADH titrations that A is appreciable (Lindman et al., 1972), which can be taken as evidence for a large number of peripheral positive groups which bind anions weakly. It is possible that some of these may function in attracting the coenzyme molecule prior to its positioning in the active site.

Computer least-squares fits of the data of Figure 3 to eq 2 yielded the values of K_L/K_{Cl} and A given in Table I and the full-drawn curves in Figure 3 correspond to these constants. If we consider the residual relaxation rate, it is evident that the ability to release Cl^- ions is highly variable; ADPR is most effective (approximately as good as NADH), while adenosine is least effective.

Titration of Native and Carboxymethylated Enzyme with the Complex Anion $Pt(CN)_4^{2-}$. $Pt(CN)_4^{2-}$ is known to bind to strong anion-binding sites of proteins in competition with halogen ions (Brändén et al., 1975). Its binding position in LADH has been determined by X-ray crystallographic studies (Eklund et al., 1976). In Figure 4 is shown the change in relaxation rate upon addition of $Pt(CN)_4^{2-}$ to native and carboxymethylated horse liver alcohol dehydrogenase, where again the full-drawn lines correspond to the constants obtained from computer least-squares fits based on eq 2, which also applies in this case.

As can be inferred from Figure 4, the decrease on addition of the anion is somewhat smaller for the caroboxymethylated enzyme than for the native, which is mirrored by the difference in A values: 4.5×10^5 s^{-1} M^{-1} for the modified enzyme and 3.9×10^5 s^{-1} M^{-1} for the native enzyme.

Influence of 1,10-Phenanthroline on Titrations of Native and Carboxymethylated Horse Liver Alcohol Dehydrogenase.

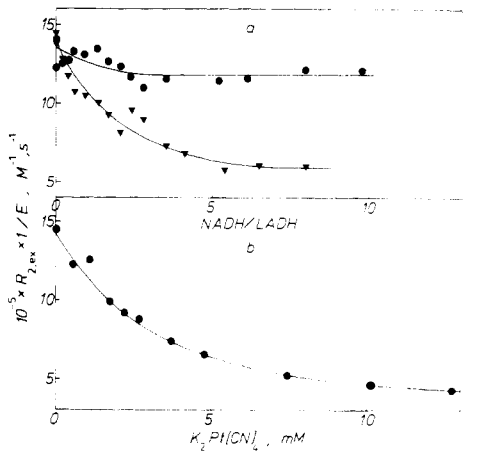

FIGURE 5: (a) Transverse excess ^{35}Cl relaxation rate $1/T_{2,ex} = R_{2,ex}$ divided by the concentration of carboxymethylated enzyme, E, as a function of the molar ratio of reduced coenzyme (NADH) to horse liver alcohol dehydrogenase (LADH). Experimental points are (▼) 83 μM carboxymethylated enzyme titrated with NADH and (●) 86 μM carboxymethylated enzyme titrated with NADH in the presence of 1,10-phenanthroline. (b) Transverse excess ^{35}Cl relaxation rate $1/T_{2,ex} = R_{2,ex}$ divided by the concentration of carboxymethylated enzyme, E, as a function of added $K_2Pt(CN)_4$ in the presence of 1,10-phenanthroline. The enzyme concentration was 86 μM. The Cl concentration was 0.5 M and the temperature 10 °C in both cases.

It has already been reported that 1,10-phenanthroline, which binds to the two active-site zinc ions of liver alcohol dehydrogenase, does not influence the Cl^- NMR line broadening caused by the native enzyme (Zeppezauer et al., 1969). This was good evidence for the absence of any contribution to ^{35}Cl relaxation from Cl^- interaction with Zn (Lindman et al., 1972).

In order to detect an anion binding to the catalytic zinc ion of the carboxymethylated enzyme, the same experiments were performed with this species. In the case where an extra anion-binding site is provided by the zinc, this effect of the carboxymethylation would presumably be eliminated through the binding of 1,10-phenanthroline to the zinc. However, since titrations with 1,10-phenanthroline gave no ^{35}Cl relaxation reduction even for the carboxymethylated enzyme, it appears that no Cl^--binding site is created on the carboxymethylation of LADH.

Rather interesting results were obtained, however, when LADH was titrated with NADH or $Pt(CN)_4^{2-}$ in the presence of excess 1,10-phenanthroline.

Both for the native and the carboxymethylated enzymes the relaxation rate reduction for NADH titrations is very much decreased in the presence of 1,10-phenanthroline, while for $Pt(CN)_4^{2-}$ no significant difference was observed. The results are shown in Figure 5 for the carboxymethylated enzyme. These findings are readily understood by assuming that there is no competition between 1,10-phenanthroline and $Pt(CN)_4^{2-}$, while there is one between 1,10-phenanthroline and NADH. As a result NADH only partly eliminates Cl^- binding. Ward & Happe (1971) have made an analogous observation for the system LADH–1,10-phenanthroline–ADPR but presented a different interpretation.

Discussion

Studies of the NMR quadrupole relaxation of halide ions have been shown to be useful as a general tool for the elucidation of anion-binding properties of proteins (Zeppezauer et al., 1969; Norne et al., 1973, 1975a,b; Lindman & Forsén, 1976; Stephens & Bryant, 1976). It has been shown that halogen NMR quadrupole relaxation measurements can be used to characterize both strong and weak anion binding sites

in proteins (Norne et al., 1975a); for example, by compeittion experiments with different anions, it was demonstrated that the relative affinities of anions to both classes of sites in human serum albumin are governed by the lyotropic or Hofmeister series, i.e., the order of increasing polarizability of the anions. As regards one class, the strong sites, these have been demonstrated to be essential for the biological function of the proteins, such as the binding of coenzymes or inhibitors (Norne et al., 1975b).

The aim of the present study was to probe in detail into the anion binding in the active site region of LADH and a number of general findings were delineated in the Results section. A general conclusion to be drawn for both the native and the carboxymethylated enzyme is that all chloride binding takes place to groups in the amino acid sequence, while there is no detectable interaction with the zinc ions. Thus 1,10-phenanthroline and other ligands known to bind to zinc have no influence on the relaxation of $^{35}Cl^-$.

The major binding site of a number of anions, e.g., $Pt(CN)_4^{2-}$, has, on the basis of the X-ray crystallographic work, been ascribed to Arg-47. The catalytic zinc ion is sufficiently close to Arg-47 so that with increasing size of the anion there should be an interdependence between anion binding at Arg-47 and ligand binding at the catalytic zinc. The present study quite well illustrates the conditions for this. Thus, in the presence of 1,10-phenanthroline, the relaxation rate decrease on addition of NADH is much smaller than in its absence.

From X-ray investigations, it is known that the coenzyme binds with the nicotinamide ribose moiety pointing to the zinc, with the ribose situated 0.6 nm from it—close enough to compete with 1,10-phenanthroline (Eklund et al., 1976). Thus, when the chelator is present, it hinders the binding of the coenzyme molecule simply by pushing it out of its position. As a consequence, other (smaller) anions, as in our case Cl^-, can now gain entrance into and bind to the anion-binding site. That the ribose ring is sufficiently close to the active-site zinc to cause steric interactions with 1,10-phenanthroline is shown by the observation that similar results as with NADH are obtained on titrating with ADPR in the presence of 1,10-phenanthroline (cf. also Ward & Happe, 1971). On the other hand, 1,10-phenanthroline has no effect on titrations with $Pt(CN)_4^{2-}$. This anion, therefore, is small enough to bind simultaneously with 1,10-phenanthroline and, as a consequence, it competes effectively with Cl . The present findings agree well with those of Gunnarsson et al. (1974) who in a study of LADH inhibition found $Pt(CN)_4^{2-}$ to bind independently of 1,10-phenanthroline.

For metalloenzymes, anion binding to the metal ion is often strong and of functional significance. In the case of LADH, several of our results tend to exclude a contribution from zinc–chloride interactions and this has also been suggested previously (Lindman et al., 1972; Ward & Cull, 1974). An additional argument based on the low value of the quadrupole coupling constant is given below. It might have been expected that the carboxymethylation of Cys-46 would have changed the character of the active site zinc enough to turn it into an effective anion-binding site. From X-ray crystallographic investigations, it has been shown that, upon carboxymethylation with iodoacetate in the crystal, the iodine binds to zinc as a fourth ligand replacing water (Zeppezauer et al., 1975). In the native enzyme, apart from the already mentioned Cys-46 and water or hydroxide ion, another cysteine and a histidine are liganded to the catalytic zinc (Zeppezauer et al., 1975). Thus, the net charge on that zinc ion is zero or minus one depending on the pH. On carboxymethylation, this charge is changed to plus one or zero which should make the metal ion more apt to interact with anions. However, these rather drastic alterations do not lead to an enhanced Cl^- binding. (Cl^- binding under conditions of very slow exchange is not detected by the present method but seems very unlikely considering results for a number of other Zn metalloproteins. See Lindman & Forsen, 1976.) The quite small changes in titration behavior caused by carboxymethylation are in line with the findings that ligands tend to bind somewhat weaker to the carboxymethylated enzyme (Reynolds et al., 1975).

An important aspect of the anion binding is the number of binding sites for different anions and their location. The present results provide a suitable basis for such a discussion and particularly significant will a correlation with the X-ray crystallographic results be. That there are at least two binding sites in the coenzyme binding region for small ions like Cl^- is implicated by several observations. Thus, the residual relaxation rate extrapolated to infinite concentration of added ligand is quite different for different cases; i.e., different ligands displace different amounts of chloride on binding to LADH. In the series of coenzyme fragments, it may be observed (Figure 3) that the amount of Cl^- release decreases along the series NADH $\simeq$ ADPR $\geq$ AMP > ADP $\simeq$ adenosine. The equality of NADH and ADPR is in line with the above presented view that Cl^- binding only occurs at a considerable distance from Zn. The fact that adenosine is competitive with Cl^- establishes that there is a second Cl^- binding site in addition to that discussed previously; the previously discussed site is situated in the region responsible for the binding of the diphosphate bridge of the coenzyme and identified as Arg-47 in correlation with X-ray findings (Norne et al., 1975b). The second site should be close to the adenosine binding pocket, presumably at Arg-271 (cf. Brändén et al., 1975). Salicylate has also been observed to bind in the adenosine binding pocket with its charged group close to Arg-271 (Einarsson et al., 1974).

The magnitude of the relaxation rate decrease also contains information on the number of binding sites. Combination of the T_1 and T_2 data gives the product of the number of bound Cl^- ions and the quadrupole coupling constant. It is significant that the best fit of the data for NADH titrations with both theoretical estimates, nuclear quadrupole resonance studies of a model system (J. A. S. Smith, personal communication), and quadrupole coupling constants obtained from ^{35}Cl quadrupole relaxation for proteins with presumably similar anion-binding sites as LADH is obtained by assuming that NADH displaces two Cl^- ions. The results from titrations with $Au(CN)_2^-$ and $Pt(CN)_4^{2-}$ (Norne et al., 1975b) showing different asymptotic values for the two ions (both differing from NADH) are also consistent with the presence of two Cl^- binding sites. According to X-ray investigations, $Au(CN)_2^-$ binds at two sites in the NADH-binding domain, one at Arg-47 and the other presumably near Arg-271, whereas $Pt(CN)_4^{2-}$ only binds at Arg-47 (Eklund et al., 1976). From observations of the partial occupancy of the sites (Söderberg et al., 1970) for these complex anions a strong site–site interaction may be inferred and this is also clearly born out in ^{35}Cl relaxation studies (Norne et al., 1975b). This resembles the cross-site interactions between Cl^- and $Au(CN)_2^-$ in the active site of carbonic anhydrase which was also demonstrated by ^{35}Cl NMR quadrupole relaxation (Wyeth & Prince, 1977). In a study of LADH inhibition (Gunnarsson et al., 1974), it was found that there are two $Au(CN)_2^-$ and one $Pt(CN)_4^{2-}$ binding sites and that there is cooperativity of the two anion-binding sites. On the other hand, this study indicated only one Cl^-

binding site but Cl^- binding to the second site is probably not strong enough to be observable under the conditions used.

Considering the ligand competition experiments of Figure 3 some further conclusions on site–site interactions may be drawn even if no strict significance can be attributed to the apparent binding constants in the presence of site–site interactions.

The values of K_L/K_{Cl} and A calculated from eq 2 are listed in Table I together with the dissociation constants obtained in phosphate buffer. K_L/K_{Cl} is a measure of the relative binding strength of L in competition with Cl^- (at 0.5 M). The most striking observation is the weak binding of ADP in 0.5 M Cl^-, which is nearly the same as for adenosine, whereas in phosphate buffer, ADP binds about 100 times stronger. The A values give a rough measure of the relative accessibility for chloride ions of the anion binding sites when the protein is saturated by ligand. These A values are found to lie between 2.3×10^5 s^{-1} M^{-1}, for ADPR which can be assumed to cover both anion-binding sites effectively, and 5.2×10^5 s^{-1} M^{-1} for adenosine, which seems to compete with chloride only within the adenosine binding pocket. The A values of the other ligands lie between these limiting values. It is interesting to note that salicylate, which is known to bind exclusively in the adenine pocket (Einarsson et al., 1974), shows a distinctly lower A value than adenosine. This shows that salicylate affects chloride binding even at Arg-47. Even Ans shows an intermediate A value. However, its mode of interaction is not well established. Thus, Einarsson et al. (1974) have presented data which show the existence of two binding sites for this ligand. It must be stressed that all the ligands except NADH and ADPR which affect both anion binding sites cannot totally prevent chloride from entering these sites. This applies also for the coenzyme fragments AMP and ADP. The latter presents a special case, as mentioned above. If only the size of the molecule is taken into account, one would expect the binding affinity to follow the series: AMP < ADP < ADPR < NADH. ADP has one more negative charge than both AMP and ADPR, which would appear to be favorable in case of a pure anionic interaction with the binding site. However, as pointed out by Frausto da Silva & Williams (1976), for understanding the anion binding to a macromolecule one has also to consider the free energy of hydration of the anions. Without going further into the treatment of these authors, it is evident that, because of the high charge density on ADP compared with that of AMP or ADPR, the former must be more strongly hydrated than the two latter ones. Thus, by considering hydration free energy, binding of ADP in the cleft near Arg-47 must be more unfavorable than binding of either AMP, ADPR, or the whole coenzyme molecule. The balance between ion–protein interactions and hydration is also certainly the explanation for our observation of the inability of orthophosphate to compete appreciably with Cl^-.

The ^{35}Cl relaxation in protein solutions generally occurs under nonextreme narrowing conditions which means that the longitudinal (T_1) and transverse (T_2) relaxation times are unequal. The relaxation equations applicable to the exchange of Cl^- with a binding site on a macromolecule have been developed by Bull (1973) and a detailed description with illustrative graphs has recently been given (Lindman & Forsén, 1976). From comparisons of the decreases in $1/T_1$ and $1/T_2$ on ligand addition, apparent correlation times and quadrupole coupling constants may be directly obtained if Cl^- exchange between the free and bound states is sufficiently rapid which applies in the present case according to variable temperature studies. From the NADH titrations, we obtain the apparent correlation time to be 9 ns and the apparent quadrupole coupling constant to be 1.7 MHz, assuming NADH to displace two Cl^- ions. (This assumption puts the quadrupole coupling constant close to that of a number of nonmetal proteins; see Bull et al. (1978).) The value of the correlation time is very much smaller than the reorientational correlation time of LADH (estimated to be 30–60 ns from the Debye–Perrin model depending on the direction of the field gradient with respect to the protein axes) and provides good evidence for the presence of very rapid internal motion at the anion binding sites. A simplified model for internal motion in macromolecules has recently been worked out (Bull, 1978; Bull et al., 1978) according to which the Cl^- ion is assumed to be bound to the end of a rigid rod which is free to diffuse about in a conical hole. An analysis of the present data on the basis of this model gives a correlation time of internal motion of 1.1 ns and a half-angle of the conical hole of ca. 73°. This would mean that the Arg groups binding Cl^- are very mobile. For another NADH-dependent dehydrogenase, lactate dehydrogenase, also extensive internal motion of arginine was demonstrated (Bull et al., 1976). The quadrupole coupling constant used in the analysis, 3.6 MHz, is quite close to that obtained in nuclear quadrupole resonance investigations of a model compound (J. A. S. Smith, personal communication). Irrespective of the mode of analysis the deduced ^{35}Cl quadrupole coupling constant of the LADH–Cl^- complex is very much lower than that expected for Cl^- interacting with Zn. So, for example, the quadrupole coupling constant is very much higher for a number of Zn–protein complexes (Norne et al., 1975b; Lindman & Forsén, 1976; Lindman, 1978) and the quadrupole coupling constant of $Na_2ZnCl_4 \cdot H_2O$ is 15–18 MHz (Irion et al., 1977).

References

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, J. E., Allison, W. S., Everse, J., Kaplan, N. O., & Taylor, S. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1968–1972.

Branden, C.-I., Jornwall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed.* 103–190.

Bull, T. E. (1973) *J. Magn. Reson. 8*, 344–353.

Bull, T. (1978) *J. Magn. Reson. 31*, 453–458.

Bull, T. E., Lindman, B., & Reimarsson, P. (1976) *Arch. Biochem. Biophys. 176*, 389–391.

Bull, T., Norne, J. E., Reimarsson, P., & Lindman, B. (1978) *J. Am. Chem. Soc. 100*, 4643–4647.

Dalziel, K. (1957) *Acta Chem. Scand. 11*, 397–399.

Einarsson, R. (1974) Dissertation, Uppsala, Sweden.

Einarsson, R., Eklund, H., Zeppezauer, E., Boiwe, T., & Brändén, C.-I. (1974) *Eur. J. Biochem. 49*, 41–47.

Einarsson, R., Widell, L., & Zeppezauer, M. (1976) *Anal. Lett. 9*, 815–823.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B. O., Tapia, O., Brändén, C.-I., & Åkeson, Å. (1976) *J. Mol. Biol. 102*, 27–59.

Forsén, S., & Lindman, B. (1978) *Chem. Br. 14*, 1, 29–35.

Fraústo da Silva, J. J. R., & Williams, R. J. P. (1976) *Struct. Bonding (Berlin) 29*, 67–121.

Gunnarsson, P.-O., Pettersson, G., & Zeppezauer, M. (1974) *Eur. J. Biochem. 43*, 479–486.

Irion, M., Weiden, N., & Weiss, A. (1977) *J. Magn. Reson. 30*, 457–468.

Li, T.-K., & Vallee, B. L. (1964) *J. Biol. Chem. 239*, 792–796.

Lindman, B. (1978) *J. Magn. Reson. 32*, 39–48.

Lindman, B., & Forsen, S. (1976) in *Chlorine, Bromine* and *Iodine NMR. Physico-chemical and Biological Applica-*

tions, Springer-Verlag, Heidelberg.
Lindman, B., Zeppezauer, M., & Åkeson, Å. (1972) *Biochim. Biophys. Acta 257*, 173–177.
Norne, J. E., Bull, T. E., Einarsson, R., Lindman, B., & Zeppezauer, M. (1973) *Chem. Scr. 3*, 142–144.
Norne, J. E., Hjalmarsson, S. G., Lindman, B., & Zeppezauer, M. (1975a) *Biochemistry 14*, 3401–3408.
Norne, J. E., Lilja, H., Lindman, B., Einarsson, R., & Zeppezauer, M. (1975b) *Eur. J. Biochem. 59*, 463–473.
Reynolds, C. H., & McKinley-McKee, J. S. (1975) *Arch. Biochem. Biophys. 168*, 145–162.
Reynolds, C. H., Morris, D. L., & McKinley-McKee, J. S. (1970) *Eur. J. Biochem. 14*, 14–26.
Rossman, M. G., Liljas, A., Brändén, C.-I., & Banaszak, L. J. (1975) *Enzymes, 3rd Ed. 11*, 61–102.
Söderberg, B.-O., Zeppezauer, E., Boiwe, T., Nordström, B., & Branden, C.-I. (1970) *Acta Chem. Scand. 24*, 3567–3574.
Stephens, R. S., & Bryant, R. G. (1976) *Mol. Cell. Biochem. 13*, 101–112.
Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.
Ward, R. L., & Happe, J. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 1444–1450.
Ward, R. L., & Cull, M. D. (1974) *Biochim. Biophys. Acta 365*, 281–284.
Wyeth, P., & Prince, R. H. (1977) *Inorg. Perspect. Biol. Med. 1*, 37–71.
Yonetani, T. (1963) *Biochem. Z. 338*, 300–316.
Zeppezauer, M., Lindman, B., Forsén, S., & Lindqvist, I. (1969) *Biochem. Biophys. Res. Commun. 37*, 137–142.
Zeppezauer, E., Jörnwall, H., & Ohlsson, I. (1975) *Eur. J. Biochem. 58*, 95–104.

Comparative Study on the Structure and Stability of Bovine Seminal Ribonuclease, Its Monomeric Bis(S-carboxymethylated-31,32) Derivative, and Bovine Pancreatic Ribonuclease†

Claudio Grandi, Giuseppe D'Alessio, and Angelo Fontana*

ABSTRACT: The secondary and tertiary structure of dimeric seminal ribonuclease, its monomeric bis(S-carboxymethylated-31,32) derivative (MCM-sRNase), and bovine pancreatic ribonuclease (RNase A) were compared by circular dichroism, difference absorption, and fluorescence techniques. The far-ultraviolet circular dichroism spectrum of MCM-sRNase is quite similar to that of RNase A and shows a minimum at 209 nm and a shoulder near 220 nm. Conversely, the spectrum of native seminal RNase is clearly different and shows a single, negative, broad trough centered at about 215 nm, reminiscent of β-structure content. The most relevant feature in the near-ultraviolet circular dichroism spectrum of seminal RNase is the absence of a positive peak near 242 nm, which instead is present in MCM-sRNase and in pancreatic RNase A. Since this positive ellipticity has been previously assigned to a contribution of exposed tyrosine residue(s) in RNase A, it is proposed that dissociation of dimeric seminal RNase into its monomeric MCM-sRNase leads to exposure of tyrosine residue(s). The deep burial of these residues in seminal RNase and their partial exposure in MCM-sRNase have been evidenced also by difference absorption, fluorescence emission, and fluorescence quenching measurements. The efficiency of quenching by added solutes in seminal RNase is much less than that observed with MCM-sRNase or pancreatic RNase A. MCM-sRNase was more sensitive than native seminal RNase and RNase A to thermal denaturation, as evidenced by monitoring the thermal unfolding of the secondary structure by circular dichroism measurements at 220 nm and of the tertiary structure by tyrosine fluorescence emission measurements. Analogously, the denaturing action of urea was more pronounced with MCM-sRNase than with native seminal RNase or pancreatic RNase A. These results indicate that the dimeric structure of seminal RNase exerts a significant protective effect toward protein denaturing agents. This effect may be physiologically significant in maintaining an effective concentration of ribonuclease in seminal fluids.

Bovine seminal ribonuclease is a dimer containing two identical peptide chains of 124 amino acid residues linked through two intermolecular disulfide bridges (D'Alessio et al., 1972a,b, 1975; Di Donato & D'Alessio, 1973). Sequence analysis of the peptide chain of seminal RNase revealed 23 amino acid substitutions compared with pancreatic RNase A,[1] but the 8 residues of half-cystine present in RNase A are observed at identical positions in the subunit chain of seminal RNase (Suzuki et al., 1976). This extensive sequence homology raises the possibility that the subunits of seminal RNase and pancreatic RNase A have similar three-dimensional structure and analogous mechanism of action. In fact, not only do the two enzymes have similar catalytic properties (Floridi et al., 1972), but in addition selective reduction of the intermolecular disulfide bridges at cystine residues in positions 31 and 32 of the polypeptide chain, followed by S-alkylation, affords a monomeric derivative of seminal RNase which is catalytically active (D'Alessio et al., 1975). This stable derivative, selectively S-alkylated seminal RNase (bis(S-

† From the Institute of Organic Chemistry (Biopolymer Research Centre, CNR), University of Padua, I-35100 Padua (C.G. and A.F.), and the Institute of Organic and Biological Chemistry, University of Naples, I-80134 Naples (G.D.A.), Italy. *Received December 21, 1978.* This research was performed with support from the National Research Council, Italy.

Abbreviations used: CD, circular dichroism; MCM-sRNase, monomeric bis(*S*-carboxymethyl)cysteine-31,32-ribonuclease (seminal); RNase A, the major component of bovine pancreatic RNase; $AcNH_2$, acrylamide; ATA, *N*-acetyl-L-tyrosinamide.

0006-2960/79/0418-3413$01.00/0