Biochimica et Biophysica Acta, 377 (1975) 1—8

BBA 67395

BINDING OF $Au(CN)_2^-$ AND $Pt(CN)_4^{2-}$ TO HORSE LIVER ALCOHOL DEHYDROGENASE

A ^{35}Cl NMR RELAXATION STUDY

T.E. BULL, BJÖRN LINDMAN, ROLAND EINARSSON and MICHAEL ZEPPEZAUER

Lund Institute of Technology, Chemical Center, S-220 07 Lund and Department of Chemistry, Agricultural College of Sweden, S-750 07 Uppsala (Sweden)

(Received August 1st, 1974)

Summary

The binding of $Au(CN)_2^-$ and $Pt(CN)_4^{2-}$ ions to the coenzyme binding site of horse liver alcohol dehydrogenase (alcohol : NAD^+ oxidoreductase EC 1.1.1.1) has been studied by ^{35}Cl nuclear magnetic relaxation. Longitudinal relaxation rates were analyzed in terms of a simple model and binding constants for $Au(CN)_2^-$, $Pt(CN)_4^{2-}$ and Cl^- were estimated. From a comparison between transverse and longitudinal relaxation rates the correlation time and the quadrupole coupling constant of bound chloride ion were obtained. The quadrupole coupling constant estimated from a simple electrostatic model for chloride ion interacting with an arginine group agrees with the experimental value.

Introduction

The last decade's advances in protein crystallography have enlarged our understanding of protein—ligand interactions. In such binding processes, most biological marcromolecules show a high degree of specificity with respect to their substrates, cofactors, effectors and inhibitors, if regarded as molecular entities. However, the binding sites are built up of many contact areas i.e. one or a few amino acid side chains with specialized purposes. Within these smaller regions we cannot characterize the interactions in terms of biological specificity but rather we must use simpler and more general physical terms. The attraction of charged groups of substrates (coenzymes, inhibitors, etc.) for example is effected by various kinds of ion binding sites, which may either contain metal ions or charged amino acid side chains. Anion binding sites very seldom show pronounced specificity for a single ionic species, but interact with many negative ions in varying degrees.

This is fortunate, since ions of different origin (physiological or non-physiological) can be used as reporter molecules at functional binding sites of macromolecules. The halogen ions have magnetic moments and (except fluoride) electrical quadrupole moments which render them useful as reporter groups for anion binding sites. Their NMR signal can be used as a titration indicator in binding studies in order to determine stoichiometry [1,2] and binding constants [3,4]. Recently it has also been pointed out [4] that the parameters derived from nuclear magnetic relaxation data can be used to characterize the ion binding (e.g. at metallic versus nonmetallic sites) and thus help to explain its effects.

The NMR studies are based on the fact that the longitudinal (T_1) and transverse (T_2) nuclear magnetic relaxation times of an ion exchanging between a site with a large probability ($P_o \approx 1$) and several sites with small probabilities ($P_i \ll 1$) may be expressed as

$$\frac{1}{T_1} = \frac{1}{T_{1o}} + \sum_{i \neq 0} \frac{P_i}{P_o} \frac{1}{T_{1i}} \tag{1}$$

$$\frac{1}{T_2} = \frac{1}{T_{2o}} + \sum_{i \neq 0} \frac{P_i}{P_o} \frac{1}{T_{2i}} \tag{2}$$

where, for a spin 3/2 ion, T_{1j} and T_{2j} are given by expressions analogous to those in ref. 5. For the case of fast exchange, the T_{1j} and T_{2j} are simply the longitudinal and transverse relaxation times at site j and are given approximately by [5]

$$\frac{1}{T_{1j}} \approx \frac{1}{10}\left(\frac{e^2qQ}{\hbar}\right)^2 (0.2\,J(\omega) + 0.8\,J(2\omega)) \tag{3}$$

$$\frac{1}{T_{2j}} \approx \frac{1}{20}\left(\frac{e^2qQ}{\hbar}\right)^2 (0.6\,J(0) + J(\omega) + 0.4\,J(2\omega)) \tag{4}$$

where

$$J(\omega) = \frac{\tau_c}{1 + \omega^2\tau_c^2} \tag{5}$$

and $e^2qQ/\hbar$ is the quadrupole coupling constant for the ion at site j, τ_c is the reorientational correlation time at site j, and ω is the resonance frequency (in rad/s).

When a reaction occurs which changes the probabilities or the relaxation times, then the resultant changes in the measured relaxation rate can be used as a titration indicator.

In order to obtain the relaxation times at a particular site, it is necessary to know the probability of that site as well as the relaxation effects from the various other sites. The problem of obtaining the probabilities has not been solved completely; however, the residual relaxation can often be determined from appropriate titration curves.

To illustrate this, we have measured the $^{35}Cl^-$-relaxation rates in aqueous

solutions of KCl in the presence of horse liver alcohol dehydrogenase and have performed titrations involving the addition of $K_2Pt(CN)_4$ and $KAu(CN)_2$. In addition, we have measured the dependence of the longitudinal relaxation rate on the chloride ion concentration. The results have been analyzed to obtain the quadrupole coupling constant and the correlation time for the chloride ion bound at the active site in liver alcohol dehydrogenase and to obtain some of the equilibrium constants associated with the various binding processes.

Experimental

The chloride relaxation times were measured at 8.82 MHz using a Bruker BKr-322s spectrometer with home made probes. The longitudinal relaxation times were measured using a $180°—t—90°$ plot method and the transverse relaxation times were measured with the Meiboom—Gill modification [6] of the Carr—Purcell sequence. All signals were averaged with a Varian 1024 CAT time average computer to obtain signal to noise ratios of at least 10 : 1. The resulting errors are estimated to be about 10%. Each point in the figures represents the average of at least two separate measurements.

The probe temperature was maintained by a stream of dry, thermostated nitrogen gas and is accurate to ± 0.5 °K.

Horse liver alcohol dehydrogenase was purchased from Boehringer Corp., Mannheim, Germany (Lot. No. 7102136). The solid material, either recrystallized or as the crystalline suspension in ethanol, was dissolved in a small volume of 0.05 M Tris—HCl buffer at pH 8.4 to which was added KCl to the appropriate chloride concentration. After centrifugation, the freshly prepared solution was diluted with the buffer containing KCl and was taken to remove the ethanol by exhaustive dialysis. For the measurements a stock solution of the enzyme was prepared and assayed according to the method of Dalziel [7]. All the enzyme solutions were freshly prepared and kept cold prior to use.

Potassium tetracyanoplatinate-II was bought from Degussa, Frankfurt, Germany and potassium dicyanoaurate-I from Alfa Inorganic, Beverly, Mass., U.S.A.

Buffer solutions were prepared from triply distilled water and ultrapure tris(hydroxymethyl)aminomethane (Tris) (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) and from ultrapure HCl and KCl (E. Merck, Darmstadt, Germany).

The titrations involving the metal cyanide complexes were performed by adding aliquots of either 0.335 M $K_2Pt(CN)_4$ or 0.227 M K $Au(CN)_2$ aqueous solutions to 2 ml of the liver alcohol dehydrogenase and KCl solution. The aliquots were measured using a 100 μl glass and stainless steel syringe.

Results and Discussion

In the analysis of our data, we will assume that each of the equilibrium constants for the association of the anions to liver alcohol dehydrogenase can be written in the form

$$K = \frac{[EX]}{[E][X]} \tag{6}$$

where [E] is the concentration of enzyme subunits not binding the anions, [X] is the concentration of free Cl^-, $Au(CN)_2^-$ or $Pt(CN)_4^{2-}$ ion, [EX] is the concentration of the anion-bound subunit. If there are two interdependent binding sites per subunit, then we will use expressions like Eqn 6, but with [E] representing the enzyme which has a ligand at one of the sites and [EX] representing the tertiary complex. In all cases, however, we will assume that bindings on the two subunits are independent.

For the case of two metal—cyanide binding sites per subunit [8—10] we will define K_1 as the binding constant for chloride ion to the free enzyme, K_2 as the binding constant for the metal—cyanide complex to the site at which the Cl^- also binds (to be called site 1), K_3 as the binding constant for the metal—cyanide complex to site 2 (not in common with the Cl^-), and K_4 as the binding constant for the chloride ion to the enzyme which has the metal—cyanide complex bound at site 2. Thus, we are explicitly assuming that site 1 cannot simultaneously bind Cl^- and the metal—cyanide complex. Furthermore, we are neglecting the possibility of simultaneous metal—cyanide complex binding to both sites 1 and 2. These assumptions are consistent with the findings of Gunnarsson et al. [9] for liver alcohol dehydrogenase in the presence of chloride concentrations in excess of 100 mM and will be shown to be consistent with our data.

With these definitions, then, it can be shown that in an enzyme solution the difference between the chloride longitudinal relaxation rate in the absence of metal—cyanide complex and its relaxation rate in the presence of a concentration [M] of the metal—cyanide complex is

$$\Delta_1 = \frac{2}{T_{1i}} \frac{E_o}{1 + K_1[Cl]} \left\{ \frac{[M](K_1K_2 + K_1K_3 - K_3K_4)}{1 + K_1[Cl] + [M](K_2 + K_3 + K_3K_4[Cl])} \right\} \quad (7)$$

where T_{1i} is the relaxation time for the chloride bound to the site excluded by the metal cyanide complex, E_o is the total concentration of the enzyme ($2E_o$ is the total concentration of subunits), [Cl] is the chloride ion concentration, and we have assumed that [Cl] and [M] are much larger than E_o and that there is one chloride ion bound per subunit. The difference in the transverse relaxation rates in the absence and presence of the metal cyanide complex, Δ_2, is given by Eqn 7, with T_{1i} replaced by T_{2i}. The advantage of this formulation is that Δ_1 and Δ_2 are independent of the residual chloride binding and depend only on the properties of the bound chloride which is excluded by the addition of the metal cyanide complex and on the various equilibrium constants.

Fig. 1 shows the experimental values for the chloride Δ_1^{-1} versus $[M]^{-1}$ for the addition of $Pt(CN)_4^{2-}$ to 0.15 mM liver alcohol dehydrogenase in 0.5 M KCl solution. Fig. 2 shows the analogous plot for the addition of $Au(CN)_2^-$ to 0.19 mM liver alcohol dehydrogenase in 0.5 M KCl solution. Clearly both titrations obey the form of Eqn 7. In this respect, it can be shown that if simultaneous binding of the metal—cyanide complex to two sites per subunit were making a significant contribution, then the plot would be non-linear. Consequently, within the experimental errors, we are justified in neglecting such simultaneous binding.

The results of X-ray diffraction studies of liver alcohol dehydrogenase—$Pt(CN)_4^{2-}$ complexes [8,9] show that $Pt(CN)_4^{2-}$ binds at only one site per

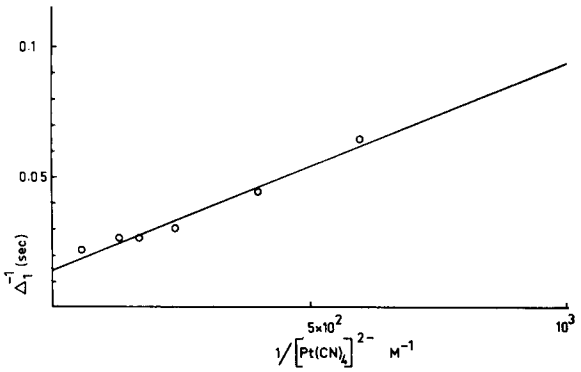

Fig. 1. Δ_1^{-1}, the inverse of the difference between the chloride longitudinal relaxation rates measured in the absence and in the presence of various concentrations of $Pt(CN)_4^{2-}$, versus the inverse of the $Pt(CN)_4^{2-}$ concentration. The liver alcohol dehydrogenase concentration is 0.15 mM, and the temperature is 6°C. The curve is a least squares fit of the data.

subunit. Consequently, we can take $K_3 = 0$ in Eqn 7 for the analysis of the $Pt(CN)_4^{2-}$ titration. Thus, the ratio of the intercept to the slope of the plot in Fig. 1 gives us $K_2/(1 + K_1\ [Cl]) = 180\ M^{-1}$. Taking K_2, the binding constant for $Pt(CN)_4^{2-}$ at 6°C and pH = 8.4, to be 8 mM^{-1} [11], we calculate a value for the binding constant of the chloride ion to site 1 to be $K_1 = 90\ M^{-1}$. This value is considerably larger than the 15—30 M^{-1} reported by Coleman and Weiner [12] for the chloride binding at 25°C and pH 7.

Using our value of K_1, the values of Δ_1^{-1} and Δ_2^{-1} extrapolated to infinite $Pt(CN)_4^{2-}$ concentration, and Eqns 3—5 and 7, we can calculate the quadrupole coupling constant and the correlation time for the chloride bound to site 1. Assuming that the $Pt(CN)_4^{2-}$ excludes two chloride ions per molecule (one per subunit), then five separate determinations at 6°C yielded e^2qQ/h = 2.5 ± 0.2 MHz and τ_c = 23 ± 11 ns, where the errors are the standard deviations. Δ_1 in the limit of infinite $Pt(CN)_4^{2-}$ concentration was found to decrease with increasing temperature, a fact which justifies the assumption of fast exchange, and at 25°C it was found that e^2qQ/h = 2.55 MHz and τ_c = 9 ns.

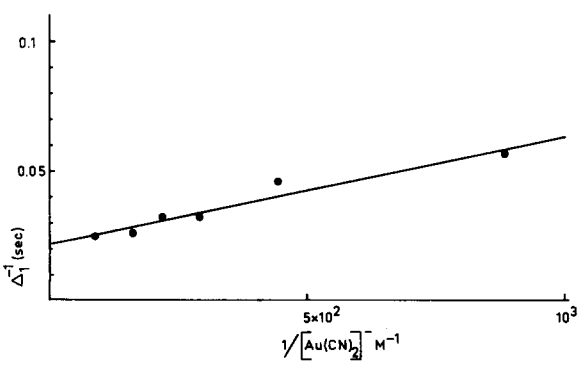

Fig. 2. Δ_1^{-1}, the inverse of the difference between the chloride longitudinal relaxation rates measured in the absence and in the presence of various concentrations of $Au(CN)_2^-$, versus the inverse of the $Au(CN)_2^-$ concentration. The liver alcohol dehydrogenase concentration is 0.19 mM, and the temperature is 6°C. The curve is a least squares fit of the data.

The quadrupole coupling constant is in good agreement with the value of about 2.5 MHz obtained for the chloride binding to hemoglobin [13] (assuming one bound chloride per subunit). Taking the difference in size between hemoglobin and liver alcohol dehydrogenase into account [13], the correlation times also agree well within the experimental errors [13]. Consequently, both the nature of the binding forces and the motion of the bound ion seem to be quite similar for the chloride binding to liver alcohol dehydrogenase and hemoglobin.

If we assume that the field gradient experienced by the bound chloride ion is due to a positively charged group on the enzyme, then the quadrupole coupling constant may be estimated from the simple electrostatic model treated by Cohen and Reif [14,15]. Assuming that the chloride ion binds to the $C(NH_2)_2^+$ group of the arginine, then for a Cl^- radius of 1.81 Å, a NH_2 radius of 1.48 Å, C—N bond lengths of 1.40 Å, and a N—C—N bond angle of 120°, we calculate that the Cl^- nucleus is 4.50 Å from the positively charged carbon atom. Taking the ^{35}Cl quadrupole moment and Hertz's value for the antishielding factor [16] (assuming the dielectric constant to be $\gg 1$), then this leads to a value of 2.77 MHz for e^2qQ/h. This calculated value should be viewed as an estimate, since the various parameters and especially the antishielding factor are not accurately known. However, the good agreement between the calculated and experimental values shows that the observed relaxation rates are consistent with the chloride ion's binding to an arginine group.

Since the relaxation rate in the presence of an excess of $Pt(CN)_4^{2-}$ is independent of the chloride ion concentration from 0.2 to 2.0 M, within the experimental error of 10%, it follows that $Pt(CN)_4^{2-}$ excludes all of the tightly bound chloride ions. Since the ions excluded by $Au(CN)_2^-$ are also tightly bound, the two cyanide complexes must exclude at least some of the same chloride ions. Consequently, we have assumed that T_{1i} is the same for both processes.

With this assumption, the value for K_1, and the titrations as a function of the $Au(CN)_2^-$ concentration and of the Cl^- concentration, we can evaluate the two remaining independent variables in Eqn 7, namely $K_2 + K_3$ and K_3K_4. Taking $K_1 = 90\ M^{-1}$ we obtain the fits shown in Figs 2 and 3 with $K_2 + K_3 = 21\ mM^{-1}$ and $K_3K_4 = 4500\ M^{-2}$. Also included in Fig. 3 are the Cl^- concentration dependence of Δ_1^{-1} for the chloride ions excluded by $Pt(CN)_4^{2-}$ and the fit obtained with $K_3 = 0$. In both cases the liver alcohol dehydrogenase concentration is 0.12 mM.

Gunnarsson et al. [10] have measured the binding constant for $Au(CN)_2^-$ to site 2 of the enzyme—chloride complex and obtained $1.6\ mM^{-1}$. In the notation of this article, their binding constant corresponds to K_3K_4/K_1. Our determination puts this value at $0.05\ mM^{-1}$. The origin of this difference is not certain, but it may be the result of our assuming too simplified a model for the $Au(CN)_2^-$ binding.

Finally we have measured the Δ_1 and Δ_2 resulting from the addition of NADH. If we assume that the relaxation time for the bound chloride is the same in the presence and in the absence of NADH and that the NADH binds completely to the available sites on liver alcohol dehydrogenase up to a mole ratio of 2 : 1 NADH : liver alcohol dehydrogenase, then we can calculate the

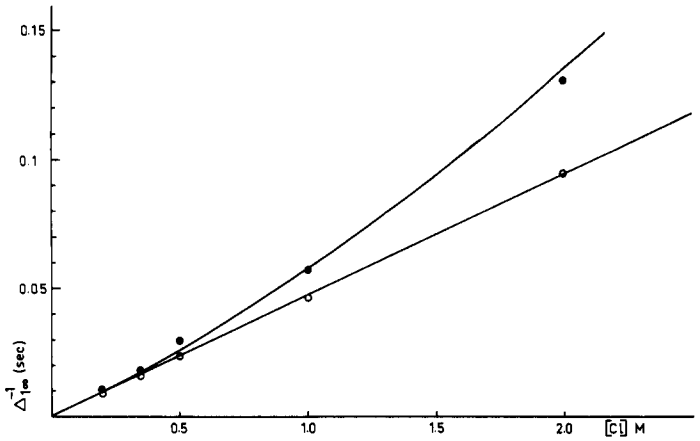

Fig. 3. $\Delta\bar{\tau}_1^{-1}{}_\infty$, the value of $\Delta\bar{\tau}_1^{-1}$ (see text) extrapolated to infinite metal–cyanide complex concentration, versus the chloride ion concentration. ○ and ● represent the values for the addition of $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$, respectively. The liver alcohol dehydrogenase concentration is 0.12 mM and the temperature is 6°C. The curves have been drawn using the parameters described in the text.

binding constant for the chloride to the $(NADH)_2$–liver alcohol dehydrogenase complex, i.e.

$$\Delta_1 = \frac{n E_o}{T_{1i}} \frac{K_5}{1 + K_5[Cl]} \tag{8}$$

where K_5 is the binding constant for Cl^- to the $(NADH)_2$–liver alcohol dehydrogenase complex and the other symbols have their usual meanings. Performing the calculations with $n = 2$, we obtain $K_5 = 1.1\ M^{-1}$. This is in agreement with Coleman and Weiner's [12] values of from 0.9–5 M^{-1}.

These results clearly show the utility of using metal–cyanide complexes to study anion binding to specific sites on macromolecules. The X-ray diffraction data enable one to determine the position of the metal–cyanide complex's binding site. Performing the appropriate titrations then enables one to determine the relaxation properties of the anion which is excluded by the metal–cyanide complex without the necessity of making assumptions about the extent of non-specific binding.

Finally, one can use these data to derive quantitative information about the nature of the anion binding site and about interactions between this binding site and molecules whose binding to the enzyme is of biochemical interest.

References

1 Gillberg-La Force, G. and Forsén, S. (1970) Biochem. Biophys. Res. Commun. 38, 137–142
2 Lindman, B., Zeppezauer, M. and Åkeson, Å. (1972) in Structure and Function of Oxidation Reduction Enzymes (Åkeson, Å. and Ehrenberg, A., eds), pp. 603–611, Pergamon Press, Oxford
3 Navon, G., Shulman, R.G., Wyluda, B.J. and Yamane, T. (1970) J. Mol. Biol. 51, 15–30
4 Norne, J.-E., Bull, T.E., Einarsson, R., Lindman, B. and Zeppezauer, M. (1973) Chem. Scr. 3, 142–144
5 Bull, T.E. (1972) J. Magnet. Resonance 8, 344–353
6 Meiboom, S. and Gill, D. (1958) Rev. Sci. Instrum. 29, 688–691
7 Dalziel, K. (1957) Acta Chem. Scand. 11, 397–398

8 Söderberg, B.-O., Zeppezauer, E., Boiwe, T., Nordström, B. and Brändén, C.-I. (1970) Acta Chem. Scand. 24, 3567—3574
9 Brändén, C.-I., Zeppezauer, E., Boiwe, T., Söderlund, G., Söderberg, B.-O. and Nordström, B. (1970) in Pyridine Nucleotide Dependent Dehydrogenases (H. Sund, ed.), pp. 129—134, Springer-Verlag, Berlin
10 Gunnarsson, P.-O., Pettersson, G. and Zeppezauer, M. (1974) Eur. J. Biochem. 43, 479—486
11 Gunnarsson, P.-O. and Pettersson, G. (1974) FEBS Lett., 43, 289—292
12 Coleman, P.L. and Weiner, H. (1973) Biochemistry 12, 1705—1709
13 Bull, T.E., Andrasko, J., Chiancone, E. and Forsén S. (1973) J. Mol. Biol. 73, 251—259
14 Cohen, M.H. and Reif, F. (1957) Solid-State Phys. 5, 321—436
15 Wennerström, H., Lindblom, G. and Lindman, B. (1974) Chem. Scr., 6, 97—103
16 Hertz, H.G. (1973) Ber. Bunsenges. Phys. Chem. 77, 531—540