PH-DEPENDENCE OF THE BINDING OF $PT(CN)_4^{2-}$, AMP, AND ADENOSINE TO HORSE LIVER ALCOHOL DEHYDROGENASE

P.-O. GUNNARSSON and G. PETTERSSON

Avdelningen för Biokemi, Kemicentrum, Lunds Universitet, Box 740, S-220 07 Lund 7, Sweden

Received 26 April 1974

1. Introduction

The binding of the coenzymes, NAD^+ and NADH, to horse liver alcohol dehydrogenase (EC 1.1.1.1) has been extensively studied [1]. One of the more interesting and still unexplained phenomena observed is the effect of pH on the stability of the binary enzyme–coenzyme complexes. The dissociation constant $K_{E,R}$ for the binding of NADH exhibits a small increase between pH 6 and 9, followed by a large increase between pH 9 and 10 [2–4]. The dissociation constant $K_{E,O}$ for the binding of NAD^+, on the other hand, decreases gradually between pH 6 and 10 [3–6]. There is strong evidence suggesting that the pH-effect on $K_{E,O}$ can be attributed mainly to an interaction between the positive charge on the pyridinium ring of NAD^+ and the pH-dependent charge of the active-site zinc ion [1,3,6], but the reason for the sharp change of $K_{E,R}$ between pH 9 and 10 has remained obscure.

It has recently been suggested that arginine-47 in the amino acid sequence of liver alcohol dehydrogenase is part of a common binding-site for negative charges on the coenzyme and coenzyme fragments such as AMP and ADP-ribose, or coenzyme-competitive anions such as $Pt(CN)_4^{2-}$ [7]. Neutralization of this positively charged amino acid residue at high pH could, possibly, be one of the main factors responsible for the drastic change in the dissociation constant for NADH between pH 9 and 10. This idea has now been tested by determination of the effect of pH on the kinetics of inhibition of horse liver alcohol dehydrogenase by $Pt(CN)_4^{2-}$, AMP, and adenosine.

2. Materials and methods

Materials and methods used in the present investigation were the same as those described in a previous report on the inhibition of horse liver alcohol dehydrogenase by coenzyme-competitive anions at pH 7 [7]. Experiments at pH 6–8 were carried out in sodium phosphate buffers of ionic strength 0.1, and 0.1 M glycine–NaOH buffer was used at pH 9–11.

Determinations of the inhibitory effect of $Pt(CN)_4^{2-}$, AMP, and adenosine were made by measurement of the enzymatic reaction velocity (v, calculated per active site of the protein) for about 25 different combinations of concentrations of substrate (0.3–10 mM ethanol) and coenzyme (3–60 μM NAD^+) at 4–6 different concentrations of the inhibitor (I), observations being fitted to the modified Dalziel relationship [8]

$$1/v = \phi_0 + \phi_2/[S] + (1 + [I]/K_i)(\phi_1 + \phi_{12}/[S])/[NAD^+] \quad (1)$$

by standard statistical procedures described elsewhere [9]. Estimates of Dalziel coefficients obtained in the absence of inhibitors (ϕ_i) agreed well with those reported previously [8,10], and no non-random deviations from a Dalziel type of rate behaviour were observed in the presence of inhibitors. The choice of the above model was based upon regression analyses showing that all inhibitors tested were strictly competitive with NAD^+ between pH 6 and 10.5. Preliminary estimates of the inhibition constant K_i were obtained from replots of the ratio of slopes of Lineweaver–Burk

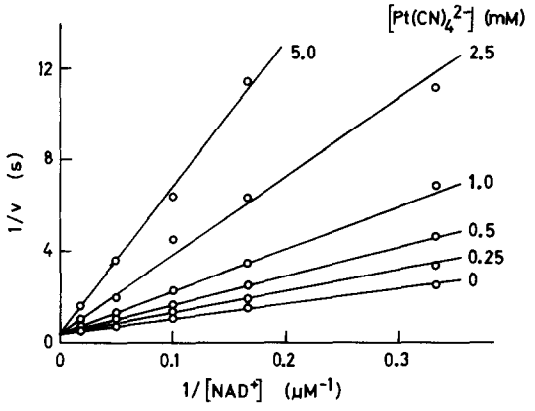

Fig. 1. Effect of $Pt(CN)_4^{2-}$ on Lineweaver–Burk plots for the oxidation of ethanol by NAD^+ in the presence of liver alcohol dehydrogenase. Reactions were performed at 25°C in 0.1 M glycine–NaOH buffer pH 10, containing 2.6 nN enzyme, 10 mM ethanol. and varied amounts of NAD^+ (3–60 μM) and $Pt(CN)_4^{2-}$ (0–5 mM).

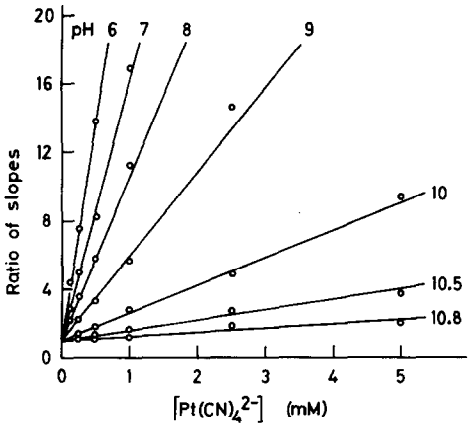

Fig. 2. Normalized replots of slopes according to Eq. (2) for the inhibition of liver alcohol dehydrogenase by $Pt(CN)_4^{2-}$ at different pH. Reactions were performed at 25°C in 0.1 M glycine–NaOH buffer (pH 9–11) or sodium phosphate buffer of ionic strength 0.1 (pH 6–8).

graphs with respect to NAD^+ vs the concentration of inhibitor, using the relationship (cf. Eq. (1)):

$$\frac{\text{Slope in the presence of I}}{\text{Slope in the absence of I}} = 1 + [1]/K_i. \qquad (2)$$

Final estimates of K_i were computed statistically [9].

3. Results

3.1. *Inhibition by $Pt(CN)_4^{2-}$*

The complex anion $Pt(CN)_4^{2-}$ has previously been shown to function as a coenzyme-competitive inhibitor of liver alcohol dehydrogenase at pH 7, yielding inhibition kinetics conforming to Eq. (1) [7]. The same rate equation was found to govern the inhibitory action of $Pt(CN)_4^{2-}$ on the enzymatic oxidation of ethanol by NAD^+ over the entire range of pH-values tested in the present investigation. This is illustrated in fig. 1 by example of the Lineweaver–Burk plots obtained at pH 10 using 10 mM ethanol, which show that the inhibition by $Pt(CN)_4^{2-}$ is strictly competitive with NAD^+ under such conditions. The linear dependence of the reciprocal reaction velocity on the inhibitor concentration at different pH is indicated by the normalized replots of slopes according to Eq. (2) given in fig. 2, from which it can be seen that the inhibition constant for $Pt(CN)_4^{2-}$ steadily increases with increasing pH between 6 and 10.8. Final estimates of K_i for $Pt(CN)_4^{2-}$ at different pH were calculated statistically by non-linear regression analysis, and the values obtained are listed in table 1.

$Pt(CN)_4^{2-}$ was, similarly, found to act as a coenzyme-competitive inhibitor of the enzymatically catalyzed reduction of acetaldehyde by NADH at pH 7 and 10, yielding inhibition constants agreeing well

Table 1
Effect of pH on dissociation constants for binary-complex formation between liver alcohol dehydrogenase and various ligands

pH	Dissociation constant in μM for				
	$Pt(CN)_4^{2-}$	AMP	Adenosine	ADP-ribose	NADH
6.0	40	35	3000	9	0.21
7.0	65	45	3500	16	0.40
8.0	100	90	3800	21	0.46
9.0	200	140	4000	36	0.99
10.0	620	430	3900	145	5.0
10.5	1600	1700	5000	–	–
10.8	4000	–	–	–	–

Standard deviations of constants for $Pt(CN)_4^{2-}$ and AMP are 8–12%, and for adenosine 14–20%. Previously reported data for ADP-ribose [12] and NADH [4] are included for comparison.

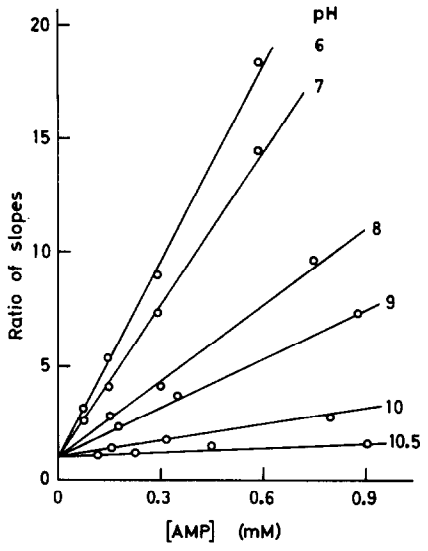

Fig. 3. Normalized replots of slopes according to Eq. (2) for the inhibition of liver alcohol dehydrogenase by AMP at different pH. Conditions as in fig. 2.

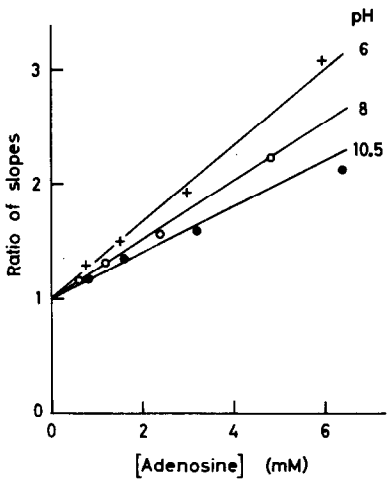

Fig. 4. Normalized replots of slopes according to Eq. (2) for the inhibition of liver alcohol dehydrogenase by adenosine at different pH. Conditions as in fig. 2.

with those determined for the reaction between ethanol and NAD^+.

3.2. *Inhibition by AMP and adenosine*

Analyses of the kinetics of inhibition of the enzymatic reaction between ethanol and NAD^+ by AMP and adenosine revealed that the latter coenzyme fragments are strictly competitive with NAD^+ at pH 6–10.5, as has previously been reported for the inhibition kinetics observed at pH 7 and 10 [11]. Fig. 3 shows normalized replots of slopes according to Eq. (2) for the inhibition by AMP at different pH, and fig. 4 the corresponding data for the inhibition by adenosine. Estimates of the inhibition constants for AMP and adenosine at different pH, obtained on fitting Eq. (1) to the experimental data, are listed in table 1.

4. Discussion

Theorell et al suggested earlier that the increase in $K_{E,R}$ at high pH might indicate that the pyridine ring in NADH is bound to an ionizing group in liver alcohol dehydrogenase with a pK around 10, possibly a sulphydryl group [1,3]. They later found that the dissociation constant for the binary complex formed between enzyme and the coenzyme fragment ADP-ribose shows a pH-dependence which is parallel with that of $K_{E,R}$, indicating that the general feature of the pH-dependence of $K_{E,R}$ can be attributed to the interaction between the protein and the ADP-ribose moiety of NADH [5,12]. The fluorescence–polarization studies of Kaplan et al. [13] and Weiner [14] lend strong support to this idea in showing that the nicotinamide ring of NADH is being held with the same degree of tightness to the protein between pH 6 and 10.

The crystallographic data reported by Brändén et al. [15] provide evidence that the ADP-ribose molecule binds in an extended conformation to liver alcohol dehydrogenase, with the adenine end lying in a hydrophobic pocket at some distance from the active-site region and the terminal ribose pointing into the active-site cleft towards the catalytically essential zinc ion. Any part of the molecule could, therefore, contribute signficantly to the stability of the binary enzyme–ADP–ribose complex, and it is not obvious a priori to what extent the different interactions between enzyme and ligand are affected by variations of the pH. The important contribution of the terminal ribose

moiety in the binding of ADP-ribose has been emphasized by Yonetani [12]. The inhibition data in table 1, however, show that the pH-dependence for the binding of AMP parallels that of the binding of ADP-ribose between pH 6 and 10, indicating that the interaction between enzyme and the terminal ribose in ADP-ribose is essentially independent of pH over the range tested. It is, similarly, evident from the data in table 1 that the effect of pH on the binding of AMP and ADP-ribose cannot be attributed to the adenosine portion of the ligands; adenosine binds weakly to the enzyme, yielding an inhibition constant that remains essentially unaffected by pH between 6 and 10.

These observations strongly suggest that the pH-dependence for the binding of AMP and ADP-ribose can be attributed mainly to interactions between the enzyme and the negatively charged phosphate group(s) of these ligands. Evidence for the presence of an anion-binding site located in the region of the protein where the phosphate group of AMP binds has been obtained kinetically [7], and is supported by crystallographic data showing that the complex anion $Pt(CN)_4^{2-}$ binds at approximately the same position in the protein as the phosphate groups of ADP-ribose [16]. The inhibition data for $Pt(CN)_4^{2-}$ reported in table 1 provide direct evidence that the anion-binding capacity of this site exhibits a pH-dependence analogous to that for the binding of AMP, ADP-ribose and NADH, and the present results seem to establish that the general feature of the variation with pH of the dissociation constant for NADH, particularly the sharp decrease above pH 9, to a major extent reflects the pH-dependence of the interaction between the anion-binding site on the protein and the phosphate groups of the coenzyme.

Arg-47 in the amino acid sequence of liver alcohol dehydrogenase has been suggested to be part of the above anion-binding site [7], and crystallographic data confirm that $Pt(CN)_4^{2-}$ and the phosphate groups of ADP-ribose are bound sufficiently close to Arg-47 to allow the formation of a salt bridge involving the guanidinium group of Arg-47 [16]. Since the reactive cysteine and lysine residues in the protein seem to have no central function in the binding of the ADP-ribose portion of the coenzyme [11,17], the present results might indicate that the drastic decrease of the dissociation constant for NADH at high pH is caused by neutralization of the positive charge on Arg-47. No firm conclusion can be drawn in this respect, however, as the anion-binding capacity of the coenzyme-binding site may be constituted by other pH-dependent factors than the presence of a single positively charged group in the protein.

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council. Thanks are due to Prof. C.-I. Brändén and collaborators for valuable discussion during the course of the investigation.

References

[1] Sund, H. and Theorell, H. (1963) in: The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K., eds.) 2nd Edn., pp. 25–57, Academic Press, London.
[2] Dalziel, K. (1963) J. Biol. Chem. 238, 2850–2858.
[3] Theorell, H. and McKinley-McKee, J. S. (1961) Acta Chem. Scand. 15, 1811–1833.
[4] Theorell, H. and Winer, A. D. (1959) Arch. Biochem. Biophys. 83, 291–308.
[5] Theorell, H. and Yonetani, T. (1963) Biochem. Z. 338, 537–553.
[6] Taniguchi, S., Theorell, H. and Åkeson, Å. (1967) Acta Chem. Scand. 21, 1903–1920.
[7] Gunnarsson, P.-O., Pettersson, G. and Zeppezauer, M. (1974) Eur. J. Biochem. 43 (1974) 479–486.
[8] Dalziel, K. and Dickinson, F. M. (1966) Biochem. J. 100, 34–46.
[9] Pettersson, G. and Pettersson, I. (1970) Acta Chem. Scand. 24, 1275–1286.
[10] Theorell, H. and McKinley-McKee, J. S. (1961) Acta Chem. Scand. 15, 1797–1810.
[11] Reynolds, C. H., Morris, D. L. and McKinley-McKee, J. S. (1970) Eur. J. Biochem. 14, 14–26.
[12] Yonetani, T. (1963) Biochem. Z. 338, 300–316.
[13] Brand, L., Everse, J. and Kaplan, N. O. (1962) Biochemistry 1, 423–429.
[14] Weiner, H. (1968) Arch. Biochem. Biophys. 126, 692–699.
[15] Brändén, C.-I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I. and Åkeson, Å (1973) Proc. Natl. Acad. Sci. U.S. 70, 2439–2442.
[16] Brändén, C.-I. (1974) Personal communication.
[17] Plapp, B. V. (1970) J. Biol. Chem. 245, 1727–1735.