The EMBO Journal Vol.2 No.5 pp.685–689, 1983

Structure of the complex of active site metal-depleted horse liver alcohol dehydrogenase and NADH

Gunter Schneider*, Hans Eklund, Eila Cedergren-Zeppezauer and Michael Zeppezauer[1]

Department of Chemistry and Molecular Biology, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden, and [1]Fachbereich 15.2, Analytische und Biologische Chemie, Universität des Saarlandes, D-6600 Saarbrücken 15, FRG

Communicated by C.-I.Brändén
Received on 3 February 1983

The complex between active site-specific metal-depleted horse liver alcohol dehydrogenase and NADH has been studied with X-ray crystallographic methods to 2.9 Å resolution. The electron density maps revealed that only the catalytic zinc ions are removed, whereas the non-catalytic zinc sites are fully occupied. A gross conformational change in the protein induced by co-enzyme binding takes place in this enzyme species despite the absence of the metal ion in the catalytic center. This circumstance is of great importance in the understanding and further analysis of the trigger mechanisms operating during the conformational transition in alcohol dehydrogenase, since the catalytic center is located at the hinge region for a domain rotation in the subunit, and the metal atom is essential for catalysis. The overall protein structure is the same as that of an NADH complex of the native zinc enzyme and the co-enzyme is bound in a similar manner. The local structural changes observed are restricted to the empty metal binding site.

Key words: alcohol dehydrogenase/metalloenzymes/conformational transition/X-ray crystallography

Introduction

Horse liver alcohol dehydrogenase (EC 1.1.1.1.) is one of the most thoroughly studied metalloenzymes; recent reviews on structural, evolutionary and mechanistic aspects of this enzyme are found in Brändén *et al.* (1975), Klinman (1981), Zeppezauer (1983) and Eklund and Brändén (1983). The dimeric enzyme contains two zinc ions per subunit. One of the zinc ions is bound at the bottom of the active site cleft by Cys 46, His 67 and Cys 174 and participates in catalysis. Although many studies have been carried out on the function of this zinc ion during catalysis, the precise mode of action of the catalytic zinc ion is still under debate (Klinman 1981; Zeppezauer, 1983). The second zinc ion, which is ligated to four cysteines, is positioned 20 Å away from the active site in a loop on the surface of the molecule close to one contact region between the subunits. This zinc ion is thought to add to the structural stability of the protein and is, therefore, often referred to as the 'structural' zinc ion (Åkeson, 1964).

The first step in the catalytic cycle of liver alcohol dehydrogenase (LADH) is the binding of co-enzyme. A conformational transition, which consists mainly of a rotation of the catalytic domain towards the co-enzyme binding domain has been shown to occur upon NADH binding (Eklund and Brändén 1979; Eklund *et al.*, 1981). The co-enzyme binds in an extended conformation. The adenine part is bound in a hydrophobic pocket of the co-enzyme binding domain and the nicotinamide ring is close to the zinc site at the bottom of the active site cleft, where catalysis takes place. A search for factors which control this large conformational change has revealed the importance of the immediate surroundings of the nicotinamide ring in the active site of the enzyme. In the complex between the enzyme and ADP-ribose (Nordström and Brändén, 1975) the enzyme is in the open conformation. This shows that the nicotinamide ring is essential for the conformational change to take place. Crystallographic studies of co-enzyme analogues have shown that analogues with chemically modified nicotinamide moieties can bind to both enzyme conformations, depending on the presence of certain zinc ligands. For example, in the ternary complex of LADH, dimethylsulfoxide (DMSO) and 3-iodopyridine adenine dinucleotide, the enzyme conformation is closed (Samama, 1979), whereas in the ternary complex of LADH, imidazole and 3-iodopyridine adenine dinucleotide, the enzyme conformation is open and the conformation of the co-enzyme analogue is different (Samama *et al.*, 1977). In complexes containing 1,4,5,6-tetrahydronicotinamide adenine dinucleotide the conformation of the protein depends on whether the zinc ligand is water or an aldehyde, e.g. *trans*-4-(N,N-dimethylamino) cinnamaldehyde (Cedergren-Zeppezauer *et al.*, 1982).

These studies demonstrate that the nature of the zinc ligand influences the positioning of the nicotinamide ring and hence, the conformational change. This raises the question as to the extent to which the catalytic metal ion itself is required for the conformational change to take place. The successful preparation of $H_4Zn(n)_2$ LADH (the enzyme, with only the catalytic zinc ions removed; n denotes the non-catalytic zinc ions) has provided a suitable system for studying the influence of the catalytic zinc ions on co-enzyme binding (Maret *et al.*, 1979). Solution studies revealed that $H_4Zn(n)_2$ LADH interacts with co-enzyme in a manner similar to the native enzyme. In particular, the pH dependence of co-enzyme binding and the absorption and fluorescence properties of the co-enzyme complexes are similar. The binding of NAD^+ is weaker, and that of NADH considerably stronger, to $H_4Zn(n)_2$ LADH as compared with the native enzyme (Dietrich, 1980). We have undertaken an X-ray crystallographic investigation of the complex between $H_4Zn(n)_2$ LADH and NADH to 2.9 Å resolution, the results of which are presented in this communication. We also describe the structural differences found in the empty zinc site for the liganded and the unliganded form of $H_4Zn(n)_2$ LADH (Schneider *et al.*, 1983).

Results

The crystals of the complex of $H_4Zn(n)_2$ LADH with NADH were triclinic, isomorphous to those obtained for most ternary complexes of the native zinc enzyme. This fact already demonstrates that the major conformational change has occurred due to co-enzyme binding, since it has been shown that the triclinic crystals always represent the closed enzyme conformation. The overall protein structure found in this complex is in fact essentially the same as in the native tri-

*To whom reprint requests should be sent.

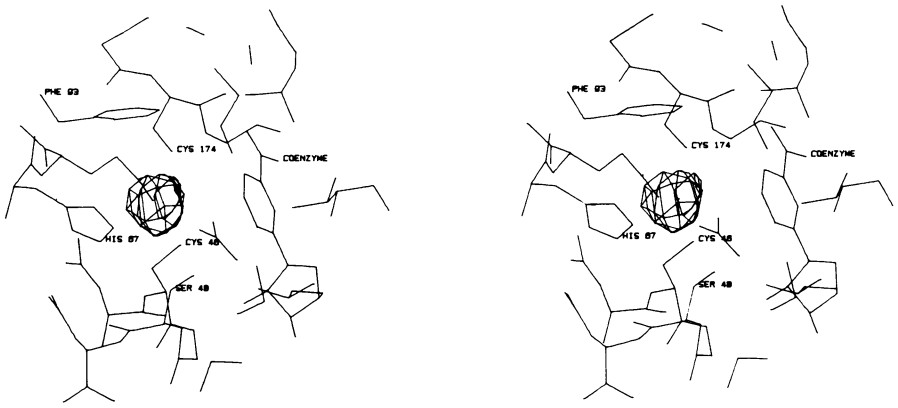

Fig. 1. Difference Fourier Map with coefficients */Fo-Fc/* at the region around the active site zinc ion. The map shows the strong minimum in electron density at the position of the catalytic zinc ion. The negative contour level shown here is four times the standard deviation of the map.

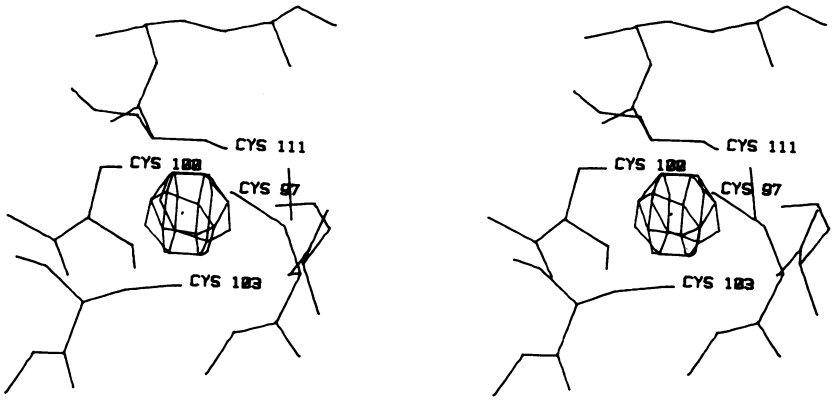

Fig. 2. Maximum in electron density at the position of the non-catalytic zinc ion in the $H_4Zn(n)_2$ LADH/NADH complex. This */Fo-Fc/* map was calculated without contributions of the zinc ions to the structure factor calculation. The contour level shown here is eight times the standard deviation of the map.

clinic ternary complex with NADH and DMSO (Eklund *et al.*, 1981). Inspection of the Fourier maps revealed only slight differences compared with the native ternary complex, which are restricted to the active site region.

The two subunits in the molecule are crystallographically independent and the structure determination gives independent results for both subunits. The results were identical in all major features for the two subunits and the differences are restricted to small details which could be due to fluctuations in the experimental background level. We therefore describe the results as relevant for both subunits.

The most prominent feature in the difference electron density map was a pronounced minimum in electron density at the position of the catalytic zinc ion (Figure 1). No change in electron density above standard deviation was found at the position of the non-catalytic zinc ion. The */Fo-Fc/* Fourier map without the contribution of the metal ions to the calculated structure factors showed a strong maximum at the position of the non-catalytic zinc ion (Figure 2). These results confirm that only the catalytic metal ions are removed, in agreement with the lack of activity and the zinc content of 1.9 mol zinc/mol LADH, as determined by atomic absorption spectroscopy. The non-catalytic zinc sites are fully occupied in the $H_4Zn(n)_2$ LADH/NADH complex. Since the crystallisation has been performed over a time scale of weeks, it can be concluded that no migration of the non-catalytic zinc ions to the metal-depleted active site has taken place.

The position of the former zinc ligands, including the main chain part of the residues 173 – 176, is slightly different. This difference is most pronounced for residue Cys 174, one of the ligands to the catalytic zinc ion in the native enzyme, which has moved towards the empty zinc binding site. Due to this movement, the sulphur atom of Cys 174 is shifted ~1 Å towards the sulphur atom of Cys 46. The side chain of this residue has a weaker density than the sulphur of Cys 174, which indicates a less rigid position. No detectable difference in the position of Cys 46 was found compared with the native enzyme complex. The sulphur-sulphur distance is thus decreased from 4.4 Å to 3.3 Å. A disulfide bridge between the two sulphurs is excluded, based on the results from the present study. This agrees with the observation that the content of thiol groups is unchanged in $H_4Zn(n)_2$ LADH (Dietrich, 1980). The imidazole ring of His 67, the third protein ligand to the zinc ion in LADH, is also slightly shifted from its original position. The shifts found in the active site region are shown in Figure 3. These shifts are in contrast to those found in the unliganded form of $H_4Zn(n)_2$ LADH. In this case, the only significant difference from the native enzyme is a rotation of the sulphur atom of Cys 46 around the C_α-C_β bond. The conformation of Cys 174 remains unchanged (Schneider *et al.*, 1983).

A strong isolated electron density was found in the hydrophobic active site pocket. This density probably corresponds to a 2-methyl-2,4-pentanediol (MPD) molecule. The crystal-

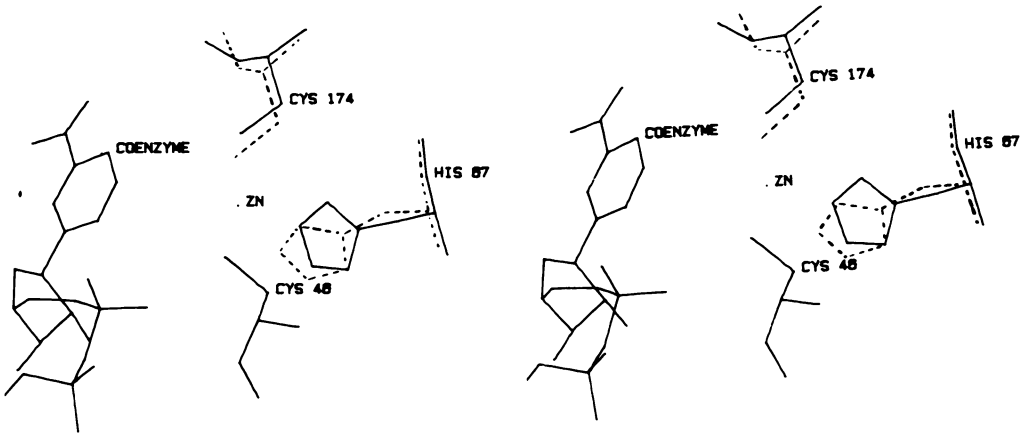

Fig. 3. Stereo diagram of the catalytic site of the $H_4Zn(n)_2$ LADH/NADH complex, compared with the ternary complex of native enzyme, NADH and DMSO. Solid lines: native ternary complex (bound DMSO not shown here); broken lines: $H_4Zn(n)_2$ LADH/NADH complex.

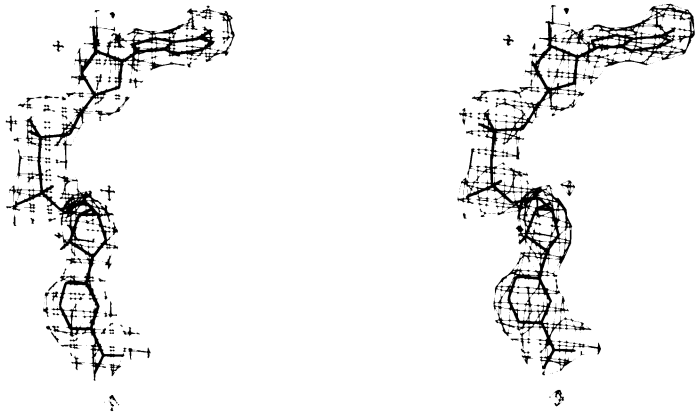

Fig. 4. Parts of a difference Fourier map with coefficients */2Fo-Fc/*, showing the electron density for the bound co-enzyme with the model of NADH superimposed. The map was computed without any contributions from the co-enzyme molecule to the structure factor calculation.

lisation mixture contained 25% MPD. Furthermore, it has been shown that MPD is bound in approximately the same position in the native enzyme (T.A.Jones, unpublished results) and in some complexes of LADH (Plapp *et al.*, 1978; Cedergren-Zeppezauer *et al.*, 1982). It is possible to fit a model of MPD into this density, causing favorable interactions with the residues of the substrate channel. The side chain oxygen atom of Ser 48 is shifted slightly by a torsion around the C_α-C_β bond and forms a hydrogen bond to one oxygen of the MPD molecule. The MPD molecule, although bound in the substrate channel, is not occupying the substrate position. It has been shown that the substrate is bound to the catalytic zinc ion at the bottom of the substrate channel (Eklund *et al.*, 1982; Cedergen-Zeppezauer *et al.*, 1982), whereas the MPD molecule in the structure described here is located further away from the catalytic center.

The binding of NADH to $H_4Zn(n)_2$ LADH was determined from Fourier maps, which were calculated without any contributions from co-enzyme atoms to the structure factor calculations. A strong, continuous electron density was found extending from the hydrophobic adenine binding pocket in the co-enzyme domain to the active site cleft. The entire co-enzyme molecule could be built in this density in an extended conformation (Figure 4). No significant deviation in the ADP-ribose part of the co-enzyme from the position observed in the LADH/NADH/DMSO complex was found. Only the nicotinamide ring was tilted a few degrees in order to obtain the best fit to the electron density. The carboxamide group of the nicotinamide ring, however, forms the important hydrogen bonds to the main chain nitrogen of residue 319 and the carbonyl oxygen of residue 317, an interaction which has been observed in all other triclinic complexes of LADH (Eklund *et al.*, in preparation). The interactions of the nicotinamide ring with the residues at the catalytic site are somewhat different as a consequence of the repositioning of the former zinc ligands, mainly Cys 174. The environments of the nicotinamide ring in $H_4Zn(n)_2$ LADH are listed in Table I. All other interactions of the co-enzyme with the protein are the same as those observed in other ternary complexes of native LADH and are described elsewhere (Eklund *et al.*, 1981).

Table I. Interactions between $H_4Zn(n)_2$ LADH and the nicotinamide ring

Atom in nicotinamide ring	Protein or MPD atom	
N1N		–
C2N	O	Val 292
	CG2	Val 292
	C6	MPD
C3N	C6	MPD
C4N	SG	Cys 174
C5N	SG	Cys 46
	SG	Cys 174
	CG1	Val 203
C6N	CG2	Val 203
C7N	CG1	Ile 318
O1N (HB)	N	Phe 319
	CZ	Phe 93
	C6	MPD
N2N (HB)	O	Ala 317
(HB)	O	Val 292
	CG1	Ile 318
	CD1	Ile 318
	CD2	Leu 309

Atoms closer than 3.8 Å to the nicotinamide ring are listed. HB indicates a hydrogen bond distance. The interactions with the bound MPD molecule are included.

Discussion

Solution studies with $H_4Zn(n)_2$ LADH (Dietrich, 1980) have shown that the optical and fluorescent properties of the protein and its complexes with NAD^+ and NADH are similar to those of the zinc enzyme. These observations suggested that the conformational change accompanying co-enzyme binding may occur even in the absence of the catalytic zinc ion. However, there are interesting quantitative differences between the native and demetallized enzyme, such as a significantly diminished quantum yield of fluorescence of the bound NADH and different rate constants for the dissociation of co-enzyme (Dietrich 1980; Zeppezauer, 1983). It has recently been shown that the structures of the co-enzyme binding domains in native and active site zinc-depleted LADH are identical (Schneider *et al.*, 1983). Thus, it was clear that essential prerequisites for the interaction between co-enzyme and the protein resulting in the conformational change had not been altered by extraction of the catalytic metal ion. However, the question remained unsolved as to whether the presence of the metal in its binding site is necessary for the conformational change to occur. Since the metal binding site is located near the rotation axis of the catalytic domain, it could be expected that the catalytic zinc ion can influence the conformational transition.

It is interesting to learn from the present X-ray study that the binding of NADH induces the same conformational change in $H_4Zn(n)_2$ LADH as it does in the native enzyme. Thus, the catalytic metal ion is not necessary for the binding of co-enzyme and the accompanying change in protein structure. A striking result is the highly similar positioning of the nicotinamide ring in the complexes of NADH and both the native and active site of the specifically metal-depleted enzyme. Obviously, the catalytic metal ion does not influence the orientation of the nicotinamide ring in the catalytic site.

Our results are different from co-enzyme binding studies using totally zinc-free LADH (Hoagstrom *et al.*, 1969; Iweibo and Weiner, 1972; Coleman *et al.*, 1972). From these studies it was concluded that, in the absence of zinc, the co-enzyme binds in a different conformation from that in the native enzyme and that the zinc ions may be necessary for the correct positioning of the co-enzyme. For evaluating the role of the functionally different zinc ions in LADH, it is essential to allocate the contributions of each metal to the observed effects. In our opinion, the totally zinc-free LADH is probably far from the native conformation, due to the removal of the non-catalytic zinc ions, indicated by the fact that no reactivation with zinc ions could be obtained by Hoagstrom *et al.* (1969). However, refolding and reconstitution of total zinc-free LADH was achieved by Rudolph *et al.* (1978) within a narrow range of Zn(II) concentrations. These and our results show that the binding of co-enzyme to the active site is dependent on the correct protein folding which, in turn, is dependent on the presence of zinc ions. It is not known whether the non-catalytic or the catalytic zinc ions, or both, are needed for the process of reassembling the protein structure. A partial unfolding of the LADH structure should influence the ability of the protein to bind the co-enzyme which may be the reason for the contradictory results.

The removal of the catalytic zinc ion, or the substitution by other divalent metal ions as Co(II), Ni(II) or Cd(II), does affect the kinetics and the thermodynamics of co-enzyme binding (Dietrich, 1980; Zeppezauer, 1983). In the case of $H_4Zn(n)_2$ LADH, this is reflected by an increased binding constant of NADH and a decreased binding constant of NAD^+. This seems plausible in view of the structural changes which take place upon removal of the metal ion from its site which is in van der Waals contact with the nicotinamide ring. The removal of the metal ion and the metal-bound water must necessarily increase the hydrophobicity of this site. Also, the thiol groups of Cys 46 and Cys 174 become protonated and therefore uncharged (Schneider and Zeppezauer, 1983). In the case of NAD^+ binding, the zinc-bound water is thought to compensate the positive charge of the NAD^+ by deprotonation yielding a hydroxyl ion. In the metal-depleted enzyme, a cysteine residue could act in a similar manner.

The presence of the zinc ion thus affects the polarity of the catalytic site. It appears as though the function of the metal ion during co-enzyme binding is to adjust the polarity of the catalytic site to provide a suitable environment for both NAD^+ and NADH binding during the catalytic cycle. This role of the catalytic metal ion may be one reason for the 10-fold decrease of the dissociation constant of NADH observed in LADH active site substituted with Cd(II) compared with the native enzyme (Dietrich, 1980); since the charge density of the Cd(II) is significantly lower due to its larger ionic radius, as compared with Zn(II).

Materials and methods

Horse LADH was obtained from Boehringer, Mannheim. NADH and dipicolinic acid are from Sigma, München. All other chemicals were of the purest grade commercially available. Double-distilled water was used throughout. All steps in the preparation of enzyme samples, including data collection, were carried out in a coldroom.

LADH was recrystallized from tert-butanol and the specific removal of the catalytic zinc ions with dipicolinic acid was performed in the crystalline state according to Maret *et al.* (1979). The specific activity of $H_4Zn(n)_2$ LADH prepared in this way, was 0.6% of that of the native enzyme; as determined by the method of Dalziel (1957). All zinc analyses were run in triplicate on a Perkin Elmer atomic absorption spectrophotometer 400. The zinc content was determined to be 1.9 mol zinc/mol $H_4Zn(n)_2$ LADH. No difference in zinc content before and after crystallisation of the binary complex was found. The crystallisation of the complex of $H_4Zn(n)_2$ LADH with NADH was achieved by dialysing a solution of $H_4Zn(n)_2$ LADH against 50 mM TES (pH 7.5) containing 0.5 mM NADH with MPD as precipitant. The details of the procedure are described elsewhere (Eklund *et al.*, 1976). The crystals were triclinic with cell dimensions a = 52.0 Å, b = 44.6 Å, c = 94.2 Å, α = 104.5°, β = 101.9° and γ = 70.7°. Data to 2.9 Å resolution were collected on a computer controlled Stoe four-circle diffractometer. Thirteen crystals were used to collect the 16 552 reflections. The procedures of data collection and data processing employed here have been described in detail elsewhere (Eklund *et al.*, 1976). The structure factors were finally scaled against the corresponding F values of the ternary complex of LADH with NADH and DMSO (Eklund *et al.*, 1981). The observed structure factors were then combined with calculated phase angles from a refined model of this ternary complex without contribution from the inhibitor. The LADH-NADH-DMSO complex has at present a crystallographic R factor of 26%. The R factor of the complex of $H_4Zn(n)_2$ LADH and NADH was 27%. Electron density maps were computed with coefficients $/2Fo\text{-}Fc/$ and $/Fo\text{-}Fc/$, where Fo denotes the observed structure factors and Fc the calculated structure factors from the refined model. Furthermore, Fourier maps, from which the contributions of the metal ions, the ligands of the catalytic metal ion (Cys 46, His 67 and Cys 174) and the co-enzyme have been excluded from the structure factor calculation, were used to detect changes in the metal distribution, the positions of the active site residues and the co-enzyme. All maps were examined in a Vector General 3404 interactive graphics display system using the FRODO program (Jones, 1978, 1982). The pictures were drawn with a Hewlett Packer plotter using a plot program written by T.A.Jones.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, grant no. 2767 from the Swedish Natural Science Research Council and by an EMBO short-term fellowship to G.S.

References

Åkeson, Å (1964) *Biochem. Biophys. Res. Commun.*, **17**, 211-214.

Brändén,C.-I., Jörnvall,H., Eklund,H. and Furugren,B. (1975) in Boyer, P.D. (ed.), *The Enzymes*, 3rd ed., Vol. XI, Academic Press, NY, pp. 103-190.

Cedergren-Zeppezauer,E., Samama,J.P. and Eklund,H. (1982) *Biochemistry (Wash.)*, **21**, 4895-4908.

Coleman,P.L., Iweibo,I. and Weiner,H. (1972) *Biochemistry (Wash.)*, **11**, 1010-1018.

Dalziel,K. (1957) *Acta Chem. Scand.*, **11**, 397-398.

Dietrich,H. (1980) Ph.D. Thesis, University of Saarbrücken, FRG.

Eklund,H. and Brändén,C.-I. (1979) *J. Biol. Chem.*, **254**, 3458-3461.

Eklund,H. and Brändén,C.-I. (1983) in Spiro,T. (ed.), *Zinc Enzymes*, J.Wiley and Sons, NY, in press.

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

Eklund,H., Samama,J.P., Wallén,L., Brändén,C.-I., Åkeson, Å. and Jones, T.A. (1981) *J. Mol. Biol.*, **146**, 561-587.

Eklund,H., Plapp,B.V., Samama,J.-P. and Brändén,C.-I. (1982) *J. Biol. Chem.*, **257**, 14349-14358.

Hoagstrom,C.W., Iweibo,I. and Weiner,H. (1969) *J. Biol. Chem.*, **244**, 5967-5971.

Iweibo,I. and Weiner,H. (1972) *Biochemistry (Wash.)*, **11**, 1003-1010.

Jones,T.A. (1978) *J. Appl. Crystallogr.*, **11**, 268-272.

Jones,T.A. (1982) in Sayre,D. (ed.), *Computational Crystallography*, Oxford University Press, NY, pp. 303-317.

Klinman,J.P. (1981), *CRC Crit. Rev. Biochem.*, **10**, 39-78.

Maret,W., Andersson,I., Dietrich,H., Schneider-Bernlöhr,H., Einarsson,R. and Zeppezauer,M. (1979) *Eur. J. Biochem.*, **98**, 501-512.

Nordström,B. and Brändén,C.-I. (1975) in Sundaralingam,M. and Rao,S.T. (eds.), *Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions*, University Park Press, Baltimore, MD, pp. 387-395.

Plapp,B.V., Eklund,H. and Brändén,C.-I. (1978) *J. Mol. Biol.*, **122**, 23-32.

Rudolph,R., Gerschitz,J. and Jaenicke,R. (1978) *Eur. J. Biochem.*, **87**, 601-606.

Samama,J.P., Zeppezauer,E., Biellmann,J.F. and Brändén,C.-I. (1977) *Eur. J. Biochem.*, **81**, 403-409.

Samama,J.P. (1979) Ph.D. Thesis, University of Strasbourg, France.

Schneider,G. and Zeppezauer,M. (1983), *J. Inorg. Biochem.*, in press.

Schneider,G., Eklund,H., Cedergren-Zeppezauer,E. and Zeppezauer,M. (1983) *Proc. Natl. Acad. Sci. USA*, in press.

Zeppezauer,M. (1983) in Bertini,I. and Drago,R. (eds.), *Function of Metals in Hydrolytic and Oxidative Enzymes*, Reidel Publishing Co., Dordrecht, in press.