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Tryptophan phosphorescence and the conformation of liver alcohol dehydrogenase in solution and in the crystalline state

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Information on the effects of crystallization upon the structure of liver alcohol dehydrogenase from horse is obtained from a comparison of the phosphorescence properties of its tryptophan residues in solution and in the crystalline state. In the crystalline state the red shift in the phosphorescence spectrum of the solvent-exposed Trp-15 attests to a decreased polarity of its environment consistent with its shielding away from the aqueous solvent probably through its involvement in an intermolecular contact. On the other hand, the triplet-state lifetime of Trp-314 which is buried deeply in the coenzyme-binding domain demonstrates that the flexibility of this region of the macromolecule is unaffected by crystallization; a conclusion supported also by the similarity in the rate of oxygen quenching of its phosphorescence. Given that lattice constraints strongly inhibit large-scale conformational changes these results allow us to identify the average solution structure with the 'open' conformer determined crystallographically.

1. Introduction

Proteins are flexible macromolecules and the free energy separating different substates is in general very small [1–5]. Therefore, when dealing with crystallographically determined structures, even if the lattice forces are weak, one is always faced with the question about how incorporation of a protein into the crystal lattice might affect its conformation [6]. Of major concern is whether the lattice forces together with the demand for perfect packing act to select one among the possible conformational states of the macromolecule in solution if not to induce a partly novel structure. In addition, because much dynamical information is also obtainable from the crystallographic approach [7], it is important to establish to what extent the flexibility of the macromolecule may be affected by lattice constraints. These questions assume even greater relevance with enzymes whose flexibility is necessary to their functioning and which during the catalytic cycle undergo large-scale structural rearrangements [4]. Based on indirect evidence, some discussions of protein structure convey the notion that in general the structure in the crystalline state is equivalent to that in solution and, with regard to the dynamical properties of the macromolecule, changes would be confined only to surface residues involved in intermolecular contacts [7–9]. By contrast, the catalytic function of enzymes is almost invariably affected by crystallization, some becoming completely inactivated [6]. Because in many instances of substrate diffusion limitations, burial of important side chains or active sites within lattice contacts can be ruled out [6], it is possible that the reasons for the altered reactivity are based on structural or dynamical perturbations of the native state.

Alcohol dehydrogenase from horse liver (LADH) is a dimeric enzyme which has been characterized in much detail in many of its functionally important complexes [10,11]. The binding

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of the coenzyme triggers the transition from an 'open' to a 'closed' configuration involving a relatively large displacement of the catalytic domain towards the coenzyme-binding domain. Since the release of the coenzyme product is the rate-limiting step of the reaction, the reverse transition from closed to open might play an important role in the overall catalytic efficiency.

LADH has two tryptophan residues per subunit possessing distinct phosphorescence spectra, the emission from the buried residue having a relatively long lifetime even at room temperature [12,13]. While spectral properties can be suitable indicators of the chemical nature of the chromophore's environment [14] the triplet-state lifetime can provide a sensitive probe of the flexibility of the protein structure [15]. In this work the phosphorescence properties of these amino acids are used to compare the conformation of the macromolecule in solution and in the crystalline state. In view of the distinct conformational states accessible to this enzyme and of the fact that lattice constraints will hinder transitions among them, we attempt to establish whether the open conformer described for the native enzyme may be identified with the average solution structure. The findings reported in this paper lead to the conclusion that except for structural changes confined to the neighbourhood of the surface residue, the dynamical properties of the macromolecule suggest that the open conformer also prevails in solution.

2. Materials and methods

LADH from horse was obtained as a crystalline suspension from Boehringer (Mannheim). 2-Methyl-2,4-pentanediol (MPD) and 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid) (Tes) were from Aldrich (Steinheim); MPD was distilled under an N_2 atmosphere prior to use.

The enzyme was dialyzed for at least 24 h against 0.03 M pyrophosphate buffer (pH 8.6). Any remaining insoluble precipitate was removed by centrifugation. The activity of LADH preparations, as measured by the method of Dalziel [16], ranged between 130 and 145%. Active-site concentrations were also determined by the spectrophotometric titration of LADH coenzyme-binding sites with NAD^+ in the presence of excess pyrazole [17]. Based on a molar extinction coefficient of $E_{280} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$ the coenzyme-binding capacity was typically 95% or better.

Crystals of LADH were grown as described by Eklund and Bränden [18]. 2 ml of enzyme solution (concentration 8–10 mg ml^{-1}) was dialyzed at 4°C against 0.05 M Tes/NH_3 buffer (pH 7.5) to which MPD was slowly added. Large crystals began to appear when the MPD concentration reached 6–9% (v/v); the final concentration of 30% was reached after 3 weeks. Subsequently crystals were sedimented by gentle centrifugation and washed twice with Tes/MPD (70 : 30, v/v) to remove crystalline debris and denatured molecules.

2.1. Sample preparation for phosphorescence measurements

To obtain reproducible phosphorescence data in fluid solutions it is of paramount importance to remove thoroughly all dissolved oxygen. Satisfactory deoxygenation was achieved by placing about 0.5 ml of the protein solution in the short arm of an L-shaped quartz cell provided with a small stirrer where gas exchange and equilibration are to take place. The solution is finally transferred to the long arm (4 mm inner diameter) for emission studies. The short arm is connected to a vacuum line for gas exchange by means of a vacuum-tight steel cap (Swagelock pat. D-316) which, upon detachment from the line, avoids air leakage. Satisfactory removal of O_2 from the solution was obtained in about 10 min by repeated application of moderate vacuum followed by inlet of very pure nitrogen (0.1 ppm in O_2, SIO, Florence) at a pressure of 3 atmospheres and gentle stirring. A check on the thoroughness of deoxygenation is provided by the dependence of phosphorescence lifetime on the amount of excitation absorbed by the sample [19].

Known concentrations of O_2 at 10°C were introduced by equilibrating the thermostatted solution for about 15 min with given partial pressures of O_2. Partial pressures were determined from the overhead pressure (digital pressure meter

OG 713 and OG 973, Officine Galileo, Florence) and the composition of an appositely prepared O_2-N_2 gas mixture (SIO, Florence). Final concentrations of O_2 were calculated using Henry's law and the solubility of O_2 in water at 10°C (Handbook of Chemistry and Physics, 41st Edition). Measurements of enzyme activity before degassing and after phosphorescence measurements showed no deterioration of the sample.

Enzyme concentrations were 2×10^{-6} M for LADH dissolved in 30:70 (v/v) MPD/Tes and about 2.5×10^{-5} M for the crystalline suspension in the same solvent. Determination of LADH concentration in equilibrium with the crystal showed that the fraction of free protein is typically 1–2%. In order to obtain clear glasses for low-temperature measurements the proportion of MPD in the solvent mixture was increased from 30 to 50%.

2.2. *Spectroscopic measurements*

Fluorescence and phosphorescence spectra were obtained with a conventionally designed instrument [19]. The excitation was selected by a 250 mm grating monochromator (Jarrel-Ash) employing a band pass of 2 nm for fluorescence and 10 nm for phosphorescence. The emission was dispersed by a 250 mm grating monochromator (Jobin-Yvon H25) and detected with an EMI 9635 QB photomultiplier. Phosphorescence decays were monitored at 440 nm by a double-shutter arrangement permitting the emission to be detected 2 ms after the excitation cut off. The decaying signal was stored, and on occurrence averaged in a Varian C-1024 time-averaging computer and successively transferred to an Apple II computer for exponential-decay analysis by a least-squares method.

The temperature in these experiments was regulated by the flow of cold nitrogen through a quartz dewar and a temperature controller (Oxford DTC2) achieving an accuracy of ± 0.2 K.

2.3. *Data analysis*

Throughout these experiments the phosphorescence decay is well represented by a single-exponential function and since the signal-to-noise ratio is good, the lifetime was obtained directly from the slope of a straight line drawn through a semilogarithmic plot of the data. Adopting this procedure the error between successive lifetime determinations in the same sample is typically 1–2%. The bimolecular quenching rate constant was obtained from measurements of the decay of phosphorescence according to the equation

$$\tau_0/\tau = 1 + \tau_0 k_q [O_2]$$

where τ_0 and τ are the phosphorescence lifetimes in the absence and presence of oxygen at concentration $[O_2]$, respectively.

3. Results

The emission spectrum of a chromophore can signal conformational changes in a macromolecule

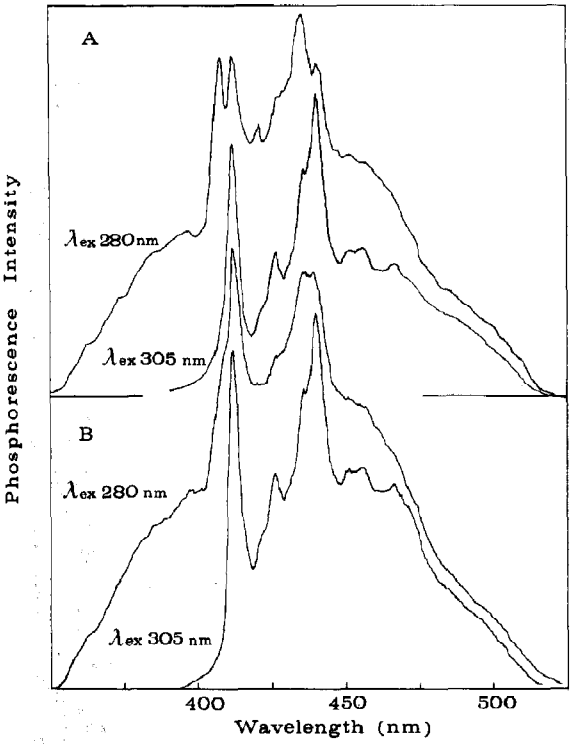

Fig. 1. Phosphorescence spectra of LADH in 50:50 (v/v) MPD/Tes buffer (50 mM, pH 7.5) at 100 K. (A) LADH in solution (2×10^{-6} M) excited at 280 and 305 nm; (B) crystalline suspension of the enzyme (2.5×10^{-5} M) excited at the same wavelengths.

undergoing crystallization if as a consequence the chemical nature of its microenvironment is altered by it. While the fluorescence of LADH in solution is practically identical to that in the crystalline phase the low-temperature phosphorescence spectra, shown in fig. 1, indicate that the environment of one of the two tryptophan residues has been modified by crystallization. In aqueous glasses, LADH Trp-15 which is solvent-exposed and Trp-314 which is buried in the coenzyme-binding domain [20] give distinct phosphorescence spectra with 0-0 vibronic bands peaking at 407 and 412 nm, respectively [12]. In the crystalline state we observe instead a single, somewhat broader 0-0 vibronic band centered at 411–412 nm with a minor shoulder at 407–408 nm whose intensity is estimated to be no more than 10% of the corresponding value in solution. By contrast, the spectrum of Trp-314 which is selectively excited at 305 nm displays sharp and superimposable vibronic bands both in solution and in the crystal. Hence, unless energy transfer from Trp-15 to Trp-314 is responsible for quenching the emission from the former, the merging of vibronic bands should be attributed to a red shift of about 4 nm in the spectrum of Trp-15. Energy transfer can be ruled out because no enhancement of the contribution of Trp-314 was found relative to when it is selectively excited nor is there any decrease in the limiting phosphorescence anisotropy. The magnitude and direction of the shift indicate a reduction in polarity of the solvation shell equivalent to going from an aqueous to a nonpolar solvent. The residual intensity at 407 nm is probably too large to be caused by the fraction of LADH molecules in solution (1–1.5%). It represents a degree of conformational heterogeneity due to molecules either on the surface corresponding to crystal defects or to the coexistence of more crystal forms.

Trp-314 is the sole residue phosphorescing in fluid solutions and an example of the time dependence of its emission with crystalline LADH is reported in fig. 2. At 10 °C the excited-triplet state decays in a strictly exponential fashion for LADH in solution and in the crystalline state. The lifetime, τ, of 1.4 ± 0.03 s is, within the approx. 2% reproducibility of the measurement, identical for the two physical states of the protein. This value

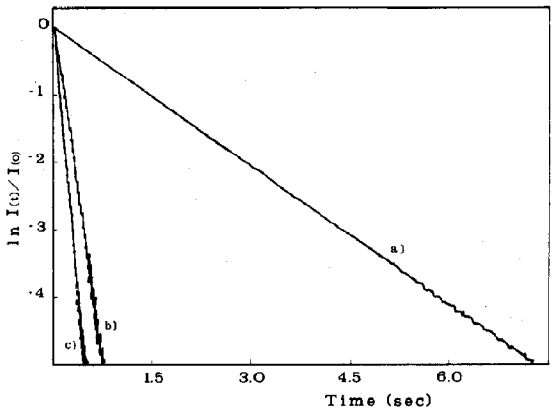

Fig. 2. Decay of phosphorescence intensity at 440 nm with time beginning 2 ms after cut off of the excitation. Samples: (a) oxygen-free suspension of crystalline LADH; (b) crystalline suspension of LADH equilibrated with an oxygen partial pressure of $P_{O_2} = 0.6$ mbar; (c) LADH in solution equilibrated with $P_{O_2} = 0.67$ mbar. Solvent, 30:70 (v/v) MPD/Tes; temperature, 10 °C.

obtained in the presence of the crystallizing agent MPD (30%) is somewhat larger than in pure buffer (1.2 ± 0.02 s) and reflects a solvent perturbation effect found also with other aliphatic alcohols. While the monoexponentiality of the phosphorescence decay implies that all macromolecules are

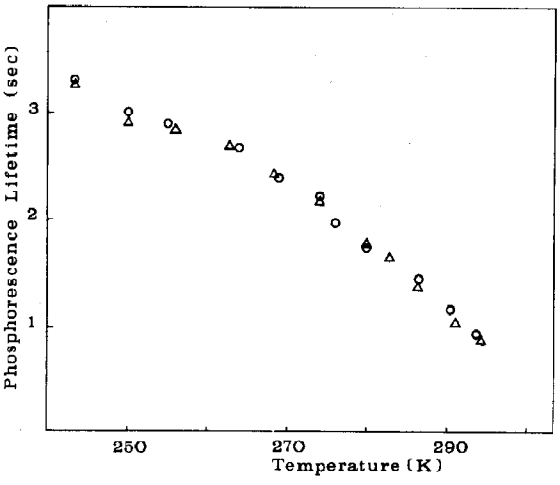

Fig. 3. Temperature dependence of the phosphorescence lifetime of Trp-314 measured at 440 nm with LADH in a crystalline suspension (○) and free in solution (△). Solvent, 30:70 (v/v) MPD/Tes.

equivalent when averaged over a time interval of the order of τ, the identity of the lifetime emphasizes that the flexibility of the coenzyme-binding domain is not affected by crystallization. That is, lattice contacts neither introduce large distortions in the protein structure nor are sufficiently strong to dampen its natural fluctuations. Further evidence on the identity between the solution and crystalline state of the enzyme is provided by the temperature dependence of τ (fig. 3) which demonstrates that the flexibility of the macromolecule is governed by the same activation parameters.

An independent view on the dynamical structure of protein molecules may be obtained from the rate at which small molecules diffuse to its inner folds [21–23]. Fig. 2 shows that the diffusion of oxygen through LADH results in the dynamic quenching of its phosphorescence. The shortening of τ with increasing partial pressures of O_2 is compared in fig. 4 for LADH in solution and in the crystalline state. With both samples we find a linear dependence of $1/\tau$ on O_2 concentration and at 10 °C the values of the quenching rate constant, k_{O_2}, obtained from the slopes of these straight lines are $8.5 \pm 1 \times 10^6$ and $6.1 \pm 0.9 \times 10^6$ M^{-1} s^{-1}, respectively. The modest 30% decrease in k_{O_2} for LADH in the crystalline state is certainly larger than the experimental error but is probably too small to imply a less flexible structure of the macromolecule. We do not know, for example, how the crystalline matrix might affect the diffusion of O_2 over the large distances, several times the dimension of the macromolecule, covered during the long triplet-state lifetime. In general, the presence of macromolecules in its pathway would act to slow down its migration. The similarity in the oxygen quenching rate constants can thus be taken as further evidence of the equivalence between the dynamic structure of LADH in solution and in the crystalline phase.

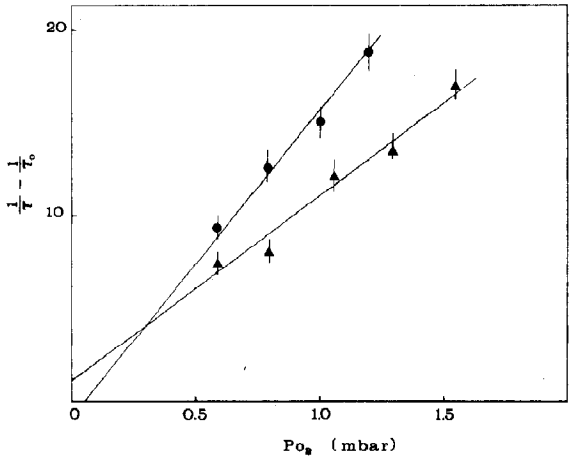

Fig. 4. Dynamic quenching of the phosphorescence of Trp-314 by oxygen in a crystalline suspension (●) and free in solution (▲). Error bars represent the range in the values obtained from three independent experiments. Solvent, 30 : 70 (v/v) MPD/Tes; temperature, 10 °C.

4. Discussion

Trp-15 is at the base of a cylinder formed by six β-strands in the βIII region of the catalytic domain and covers the open end towards the solution [24]. One side of the aromatic ring system is well exposed to the solvent whereas the other faces the hydrophobic core within this cylinder. In general, neighbouring molecules in a crystal lattice make contact with one another at only a few points on their surfaces and among surface residues only those participating in these interactions seem to be affected in their conformation, mobility and environment [7]. Within this framework we can rationalize the change in environment of Trp-15 upon crystallization, from aqueous to hydrophobic, if this residue is involved in an intermolecular contact whereby the aqueous solvent is replaced by a hydrophobic region of the neighbouring molecule.

While Trp-15 reports on perturbations confined to surface residues of the macromolecule, the location of Trp-314 deep within the coenzyme-binding domain may monitor conformational changes that could affect the core of the protein molecule. The sensitivity of the phosphorescence lifetime to the flexibility of the structure surrounding the chromophore has on other occasions revealed even subtle and otherwise undetectable structural changes in LADH, changes which were directly related to the functional state of the enzyme [15,25]. The equivalence in the kinetics of phosphorescence emission and rate of oxygen quench-

ing reported above for LADH in solution and in the crystalline state provides evidence that few if any structural and dynamical perturbations are introduced by crystallization.

LADH undergoes a major conformational change upon binding NAD^+ and NADH which can be described as a rigid body rotation of the catalytic domains with respect to the core of the molecule, thereby narrowing the clefts separating them from the coenzyme-binding domains [10]. The two structures are referred to as the open and closed conformations of the enzyme. Recent crystallographic studies have shown that depending upon the substrates, complexes with different intermediate structures can also be formed [11]. A question which carries important mechanistic implications with enzymes undergoing major rearrangements in structure with the binding of ligands is whether the conformation attained in the complex is achieved only subsequent to binding (induced fit) [26,27]. If we assume that the crystal traps the open conformer exclusively and that the transition from the open to closed conformation is slow relative to the phosphorescence decay, then we have the equality $\tau(\text{open}) = \tau(\text{crystal}) = \tau(\text{solution})$, i.e., we do identify the open conformer with the structure in solution as well. These assumptions are reasonable because there is evidence that the constraints placed by the crystal lattice on the open to closed transition are rather severe. The closed conformer crystallizes in a different crystal form and diffusion of the coenzyme into crystals of the native protein causes them to break up [28], a sign that the open to closed transition cannot be accommodated in the same crystal form.

If LADH in solution is in rapid equilibrium between open and closed conformers, the presence of the latter, which is characterized by a distinct phosphorescence lifetime (crystals of LADH · NAD^+ and LADH · NAD^+ · pyrazole in which the enzyme is in the closed configuration have $\tau =$ 50–60 ms; unpublished data) would be manifested by a shortening of τ. From the strict exponentiality of the phosphorescence decay we observe that the two conformers must interconvert rapidly relative to τ. Under these conditions the phosphorescence lifetime in solution is governed by the relation $1/\tau(\text{solution}) = f_o/\tau(\text{open}) + f_c/\tau(\text{closed})$ where f_o and f_c are the fractions of LADH molecules in the open and closed conformers, respectively [29]. Using $\tau(\text{open}) = 1.4$ s and $\tau(\text{closed}) =$ 60 ms a 2% error in $\tau(\text{solution})$ corresponds to $f_c = 10^{-3}$. If intermediate states in the pathway from the open to closed conformation were characterized by very short lifetimes then f_c would be even smaller. From this analysis we draw the conclusion that the open conformer does represent by far the most stable conformational state of LADH in solution and that the closed form is probably induced only subsequent to interactions with the coenzyme.

The identity of τ and the similarity in k_{O_2} for LADH in the two phases are manifestations that crystallization does not really dampen structural fluctuations in the macromolecule even if large amplitude rearrangements are severely limited. Incidentally, the comparison of k_{O_2} obtained in solution and in the crystalline phase also carries interesting implications regarding the mechanism by which internal tryptophans in proteins are quenched by small solutes [30]. According to the unfolding model, quenching occurs when the chromophore is temporally exposed to the solvent by a transient, partial unfolding of the polypeptide. In the penetration model small-amplitude local fluctuations in structure are sufficient to allow the migration of the quencher to the chromophore's site. The similarity in k_{O_2} for LADH in the two phases rules out the unfolding mechanism in that the constraints imposed by the crystal lattice on the structural rearrangement required to expose Trp-314 to the solvent would be prohibitive thereby reducing dramatically the quenching efficiency.

References

1 H. Frauenfelder, G.A. Petsko and D. Tsernoglou, Nature 280 (1979) 558.
2 F.R.N. Gurd and T.M. Rothgeb, Adv. Protein Chem. 33 (1979) 73.
3 M. Karplus and J.A. McCammon, Annu. Rev. Biochem. 53 (1983) 263.
4 W.S. Bennet and R. Huber, CRC Crit. Rev. Biochem. 15 (1984) 291.

5 S.W. Englander and N.R. Kallenbach, Q. Rev. Biophys. 16 (1983) 521.
6 J.A. Rupley, in: Structure and stability of biological macromolecules, eds. S.N. Timasheff and G.D. Fasman (Dekker, New York, 1969) p. 291.
7 D. Ringe and G.A. Petsko, Prog. Biophys. Mol. Biol. 45 (1985) 197.
8 T.L. Blundell and L.N. Johnson in: Protein crystallography (Academic Press, New York, 1976) p. 127.
9 B.W. Mathews in: The proteins, vol. 3, eds. H. Neurath and R.L. Hill (Academic Press, New York, 1977) p. 403.
10 H. Eklund and C.-I. Brändén in: Zinc enzymes, metal ions in biology, vol. 5, ed. T. Spiro (Wiley, New York, 1983) p. 123.
11 E.S. Cedergren-Seppezauer, in: Zinc enzymes, PBB, vol. 1, eds. I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Birkhäuser, Boston, 1986) p. 393.
12 R.M. Purkey and W.C. Galley, Biochemistry 9 (1970) 3569.
13 M.L. Saviotti and W.C. Galley, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 4154.
14 W.C. Galley, in: Biochemical fluorescence: concepts, vol. II, eds. R.F. Chen and H. Edelhoch (M. Dekker, New York, 1976) p. 409.
15 G.B. Strambini and M. Gonnelli, Chem. Phys. Lett. 155 (1985) 196.
16 K. Dalziel, Acta Chem. Scand. 11 (1957) 397.
17 H. Theorell and T. Yonetani, Biochem. Z. 338 (1963) 537.
18 H. Eklund and C.-I. Brändén, J. Biol. Chem. 254 (1979) 3458.
19 G.B. Strambini, Biophys. J. 43 (1983) 127.
20 C.-I. Brändén, H. Jornvall, H. Eklund and B. Furungen, in: The enzymes, vol. 11A, ed. P.D. Boyer (Academic Press, New York, 1975) p. 103.
21 J.R. Lakowicz and C. Weber, Biochemistry 15 (1976) 672.
22 M.R. Eftink and C.A. Ghiron, Biochemistry 12 (1973) 4171.
23 G.B. Strambini, 52 (1987) Biophys. J. 23.
24 H. Eklund, B. Nordström, E.S. Zeppezauer, G. Soderlund, I. Ohlson, T. Boiwe, B.O. Tapia, C.I. Brändén and Å. Åkesson, J. Mol. Biol. 102 (1976) 27.
25 G.B. Strambini and M. Gonnelli, Biochemistry 25 (1986) 2471.
26 H. Eklund, J.-P. Samama and T.A. Jones, Biochemistry 23 (1984) 5982.
27 M. Zeppezauer, in; Zinc enzymes, PBB, vol. 1, eds. I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Birkhäuser, Boston, 1986) p. 417.
28 T. Yonetani and H. Theorell, Arch. Biochem. Biophys. 100 (1963) 554.
29 W.R. Laws and L. Brand, J. Phys. Chem. 83 (1979) 795.
30 B. Somogyi, J.A. Norman and A. Rosenberg, Biophys. J. 50 (1986) 55.