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Spectral Observations Concerning the Binding of Zinc in Liver Alcohol Dehydrogenase

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The ultraviolet absorption spectrum of 1,10-phenanthroline when bound to liver alcohol dehydrogenase has been measured and compared with measurements made of the spectrum of 1,10-phenanthroline bound to zinc in various complex ions. In all cases the spectra are qualitatively similar, but difference spectra reveal quantitative differences in extinction coefficients and slight frequency shifts. These changes were studied as a technique for determining the nature of the binding of zinc in the enzyme by finding the model complex having the same spectral effects on 1,10-phenanthroline as does the enzyme. As a means of checking the method, a thorough study of the spectra of the mono-, bis-, and tris-1,10-phenanthroline-Zn(II) complexes was made. From these, three independent spectral criteria were adopted for distinguishing between the spectra of 1,10-phenanthroline in the three complexes. Two of these criteria showed that the enzyme-1,10-phenanthroline complex was spectrally identical to the tris-1,10-phenanthroline-Zn(II) complex but different from either the mono, or bis complex. All three spectral criteria showed the enzyme-phenanthroline complex to be indistinguishable from phenanthroline bound to $(imidazole)_{3\text{ or }4}Zn^{++}$. When the ligands glycine, L-cysteine or L-histidine were studied, the complexes formed differed significantly from those formed with the enzyme. It is concluded that on the basis of the spectral criteria developed, three or four imidazole ligands provide the best model for the environment of the zinc atom in liver alcohol dehydrogenase.

An important but difficult problem has been the elucidation of the active site of an enzyme. For metalloenzymes it is hoped that the presence of a metal atom might provide the means necessary to learn about the nature of the active site. To date all experimental evidence concerning liver alcohol dehydrogenase (LADH) is consistent with the belief that a zinc atom is included at the active site. Unfortunately such zinc atoms have neither spectral nor magnetic properties which allow direct study. The present research is directed toward a spectral study by indirect means of the nature of the environment of the zinc in the enzyme.

Vallee and Coombs[1] have shown that it is possible to measure the binding of

the ligand 1,10-phenanthroline (OP) to the enzyme by measuring changes in the ultraviolet absorption spectrum of the ligand. The spectral changes which occur on binding are quite similar, although not identical, to the spectral changes which a molecule of 1,10-phenanthroline undergoes when bound to Zn^{++} in aqueous solution. We have found that as subsequent phenanthroline molecules are added to the zinc ion there are small spectral shifts and intensity changes. Thus, the spectrum of the phenanthroline molecule when bound to a Zn atom is influenced by the nature of the other ligands which are also bound to the zinc – *i. e.*, it matters whether the zinc is bound to other phenanthrolines or whether it is bound only to water molecules of the solvent. By studying the spectrum of 1,10-phenanthroline when bound to liver alcohol dehydrogenase and comparing the results with those for 1,10-phenanthroline bound to Zn^{++} in various complexes, it should be possible to learn about the environment of the zinc ion in the enzyme. Specifically, it might be learned whether the zinc is octahedrally or tetrahedrally bound, how many points of attachment there are between zinc and the enzyme, and something about the chemical nature of these binding groups. In hope of gaining information on these points, the present research was undertaken. As model complexes for comparison with the enzyme, zinc ion was bound to the following ligands, in addition to 1,10-phenanthroline: glycine, L-cysteine, L-histidine, and imidazole.

EXPERIMENTAL

Reagents: The following chemicals were used without further purification: 1,10-phenanthroline (OP) and zinc perchlorate $Zn(ClO_4)_2 \cdot 6H_2O$, G. Frederick Smith Chemical Co.; L-cysteine (free base) and L-histidine (free base), Nutritional Biochemical Corp.; glycine (ammonia-free) and imidazole, Eastman Kodak Co. Horse liver alcohol dehydrogenase (LADH) was obtained from Worthington Enzyme Co. Water used in preparation of the enzyme solutions and in all the reagents added to the enzymes was distilled and then passed through a Barnstead demineralizer and organic material remover.

Instruments and instrumental conditions: All solutions were buffered at $pH = 6.50 \pm 0.02$ with a KH_2PO_4-NaOH buffer. All spectral measurements with the exception of those measuring the concentration of OP were made with a Cary 14 Recording Spectrophotometer. In the enzyme solution measurements, an expanded scale attachment was used that permitted recorder full scale measurements of the optical density (O. D.) in the 0.00 – 0.10 range, and the cell compartment was thermostated at $25.0 \pm 0.2^{\circ}C$. All other measurements were made at room temperature. Determinations of the OP concentrations were made on a Beckman Model DU Spectrophotometer. Matched quartz cells of 1.0 mm, 1.0 cm, 5.0 cm, and 10.0 cm path length were used to allow optical density measurements in the range 0.20 – 0.80.

Solution preparation: A standard solution of *ca.* 3.0×10^{-2} M Zn^{++} was prepared gravimetrically. The exact molarity of the Zn^{++} was determined by a ethylenediamine-NNN'N'-tetraacetic acid titration[2]. Solutions of glycine, OP, L-cysteine, L-histidine and imidazole were prepared by standard gravimetric and volumetric technique. The concentrations of the OP solutions were checked by measuring the optical densities at 2925 Å and using the recorded value $E_{2925A} = 6800$ cm²mole^{-1} liter^{-1} [3].

Procedure for ZnOP complexes: One liter OP solutions were prepared with concentrations varying at 0.5×10^{-4} M intervals from 0.5×10^{-4} to 18×10^{-4} in OP. 5.0 ml of distilled water were added to one 500 ml flask and 5.0 ml of the standard zinc solution were added to another. These were diluted to the 500 ml mark with one of the OP solutions. A difference spectrum for the region 2600 Å – 3700 Å was obtained by measuring the ZnOP complex solution versus the OP-H_2O solution of identical OP concentration. This was repeated for each OP concentration. As a check against hidden errors from continuously increasing the concentration of OP, which itself absorbs in part of the spectral region observed, a series of experiments was

Table 1. Spectral results. For the first fourteen entries in the table the $[Zn^{++}]=3.02\times10^{-4}$ M. For all other entries (exclusive of enzyme) the $[Zn^{++}]=3.08\times10^{-4}$ M. OP means 1,10-phenanthroline.

$OP/_{Zn^{++}}$	Other $lig/_{Zn^{++}}$	Position *ca.* 2940 Å peak	Position *ca.* 2725 Å peak	$\frac{O.D._{3275}}{O.D._{3440}}$
0.346		2930	2713	2.58
0.663		2930	2712	1.88
1.040		2932	2714	1.60
1.330		2935	2717	1.54
1.66		2938	2721	1.42
1.960		2937	2720	1.30
2.26		2939	2721	1.29
2.60		2942	2721	1.21
2.75		2941	2724	1.17
3.23		2943	2725	1.10
3.55		2944	2726	1.17
3.78		2943	2725	1.13
4.17		2944	2727	1.16
4.49		2944	2727	1.13
4.81		2942	2727	1.13
5.20		2943	2726	1.11
5.85		2943	2729	1.11
0.975	1.95 His	2934	2713	1.65
0.975	1.95 Cys	2932	2714	1.71
0.975	1.95 Gly	2932	2715	1.68
0.975	2.90 Imid.	2935	2715	1.68
0.975	31.4 Imid.	2936	2716	1.58
0.975	31.4 Imid.	2943	2723	1.34
Excess	Enzyme	2943	2725	1.36

done in which a constant concentration of OP was maintained and the concentration of Zn^{++} was varied. The two methods gave equivalent spectra.

Procedure for enzyme solutions: Two 10 mg samples of lyophilized LADH were placed in two 10 ml flasks. One of the samples was dissolved in cold, buffered 1.5×10^{-4} M OP and its spectrum was taken versus an OP reference solution of the same concentration. The other sample was dissolved in buffer and measured with the buffer as a reference. The spectrum of the second sample was subtracted from that of the first with proper linear adjustment for slightly differing enzyme concentrations.

Procedure for mixed ligand solutions: One liter solutions which were 3.0×10^{-4} M in OP and contained either L-cysteine, L-histidine, glycine or imidazole as the second ligand were prepared by standard gravimetric and volumetric techniques. The zinc complexes and their spectra were prepared in the same way as the ZnOP solutions.

RESULTS

Representative results of these experiments are summarized in Table 1. About half of the total observations made are quoted in the table; omitted are duplicates and points in the ZnOP system at various intermediate ratios. In all cases results are in good agreement with those quoted. The ZnOP spectrum has absorp-

tion peaks at *ca.* 2725 Å, *ca.* 2940 Å, 3275 Å, 3425 Å and shoulders at *ca.* 2990 Å, 3125 Å, 3192 Å, and 3340 Å. The *ca.* 2725 Å peak and the *ca.* 2990 Å, 3192 Å, and 3340 Å shoulders are previously unreported. Three definitive changes in this spectrum were noted on increasing the [OP]/[Zn^{++}] ratio. These were bathochromic shifts of the *ca.* 2725 Å and *ca.* 2940 Å peaks and a decrease in the ratio $O.D._{3275A}/O.D._{3425A}$. The *ca.* 2725 Å peak shifted by 16 Å and the *ca.* 2940 Å peak shifted by 14 Å. For an individual measurement the possible error in the peak position introduced by the recorder drive was ± 2 Å. A much smaller error in the peak position is anticipated in the series of ZnOP measurements. The error in the ratio of the optical densities is seen from the tabulated data to be at most 3 %. From Table 1 it is noted that the spectrum of the LADH-OP solution corresponded to an OP/Zn^{++} ratio greater than or equal to three for both of the peak shift criteria, but to an OP/Zn^{++} approximately equal to two for the third criterion, the ratio of the optical densities. Spectra of solutions which had ligand/Zn^{++} ratios of 2:1 (where the ligand is L-cysteine, L-histidine, glycine or imidazole) and OP/Zn^{++} ratios of 1:1 were identical to spectra of solutions which contained only OP in a 1:1 ratio with the Zn^{++}. All the ligands with the exception of the imidazole have stability constants of approximately equal or greater magnitudes than OP[4]. Since the spectra showed that the 1,10-phenanthroline was bound, it seems most likely that a mixed complex must be formed containing both ligands. Because of the smaller stability constants of the zinc-imidazole complexes it was necessary to prepare solutions having imidazole/Zn^{++} ratios of 31.4 and 314 also containing OP in a 1:1 ratio with the zinc. This was in order to insure the formation of mixed complexes containing at least three imidazoles and one OP. The spectrum of the mixed complex that had a 31.4 imidazole/Zn^{++} ratio corresponded to that of the 4/3 OP/Zn^{++} complex. The spectrum of the solution with an imidazole/Zn^{++} ratio of 314 was identical to that of the LADH-OP spectrum on the basis of all three criteria.

CONCLUSIONS

The subject of ligand-ligand interactions is one which has not been studied sufficiently, in the past, to allow a decision as to why the spectrum of a phenanthroline molecule bound to a zinc ion is a function of the nature of other ligands bound to the zinc. At least two explanations seem possible. Zinc is an atom which can assume either coordination number 4 or 6. Since its actual coordination number depends upon the ligand to which it is bound and the nature of the ligand, changing the coordination number would be expected to change the absorption spectrum of a bound ligand. Even if introduction of a new ligand did not cause a coordination number change, the presence of the new ligand could cause a spectral change in other ligands by the following mechanism. The new ligand would differ from the ligand it replaced in terms of the amount of electron charge displaced from it to the Zn. This in turn would influence the charge distribution between the Zn^{++} and all the other ligands. Hence the spectrum of each ligand should be a function of all the ligands present.

A comparison of the spectrum of phenanthroline bound to LADH to that of the mono-, bis-, and tris-phenanthroline Zn^{++} complexes shows that the LADH-

OP spectrum is most like that of the tris complex. This observation could be taken to indicate that the zinc in the enzyme is most probably bound octahedrally and by at least three points of attachment to the enzyme. This picture is in agreement with that proposed by Theorell based on kinetic results[5].

In order to learn more about the identity of the groups binding the zinc atom to the enzyme, the spectra of the other model complexes are helpful. Because the stability constants for zinc complexes with glycine, histidine and cysteine exceed or are approximately equal to those for phenanthroline and since they were present in a concentration double that of the phenanthroline, it seems unlikely that the phenanthroline would be able to displace any of these ligands from the zinc. If in fact it does not, then the mixed complex should be octahedral, and the spectral results indicate that none of these ligands furnishes an environment like that in the enzyme. The result with cysteine is especially interesting in this regard since it has been postulated that the strong binding of zinc in the enzyme is due to its being bound to sulfur[5]. The only model complex to exactly match spectrum with that furnished by the enzyme is the imidazole complex. This result along with the close agreement with the tris-phenanthroline complex might indicate that the zinc in liver alcohol dehydrogenase is bound to three or four imidazole residues.

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