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Resonance Raman Investigation of an Enzyme–Inhibitor Complex†

James T. McFarland,* Kenneth L. Watters, and Richard L. Petersen

ABSTRACT: The resonance Raman spectrum has been recorded for two different binary complexes formed between 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (zincon) and liver alcohol dehydrogenase. The shifts in the zincon spectrum upon complexation with enzyme in one complex are similar to those in model compounds containing azo or formazyl linkages upon complexation of these with zinc. The results are interpreted in terms of complexation of zincon to a zinc atom at the enzyme active site. Since zincon is a coenzyme competitive inhibitor, it is probably bound at or near the coenzyme binding site; the results of this study, therefore, are useful in understanding the chemistry of zinc at the enzyme active site.

The method of resonance Raman spectroscopy has recently received attention as a means of obtaining the vibrational spectra of biochemical systems with the attendent wealth of bonding and structural information inherent to vibrational spectroscopy. The potential of the resonance Raman method for answering important questions concerning chemical bonding in a molecule derives from the fact that resonance Raman spectra can be obtained in solution at concentrations of 10^{-5} M or less for samples as small as 50–100 μl and that water, as solvent, interferes minimally in the spectrum. The resonance Raman spectrum results from enhancement of Raman band intensities, often by several orders of magnitude, due to coupling of Raman active vibrations with electronic transitions of the molecule being studied. Such a spectrum can thus be obtained when the Raman excitation frequency is near an electronic absorption band for the molecule. Several macromolecules which contain chromophores in the visible region have already been studied by the resonance Raman method (Dunn *et al.,* 1973; Salmeen *et al.,* 1973; Spiro and Strekas, 1974). Noncovalent interactions of the chromophore Methyl Orange with bovine serum albumin and of a hapten–antibody complex have also been reported (Carey *et al.,* 1972; Carey *et al.,* 1973). However, there has been no report to date of the use of this technique to study the interaction between an enzyme and a chromophore known to bind reversibly at the active site of the enzyme.[1] The bound chromophore might serve as an excellent probe of the active site *via* changes in its Raman spectrum upon complexation, thus providing specific information about geometry and binding sites of the enzyme. For this study, we have chosen as our resonance Raman probe the molecule, 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (zincon) (Figure 1a). This dye molecule is known to form a 1:1 complex with zinc (Rush and Yoe, 1954) by coordination through the azo bond and N-4 of the formazyl system and through carboxylate and phenol oxygens (Figure 1b). We report here a resonance Raman study of the binding of zincon to liver alcohol dehydrogenase.

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[1] Since submission of this manuscript, it has been brought to our attention that recent Raman experiments have been performed on acylchymotrypsin (Carey and Schneider, 1974), an irreversibly bound chromophore.

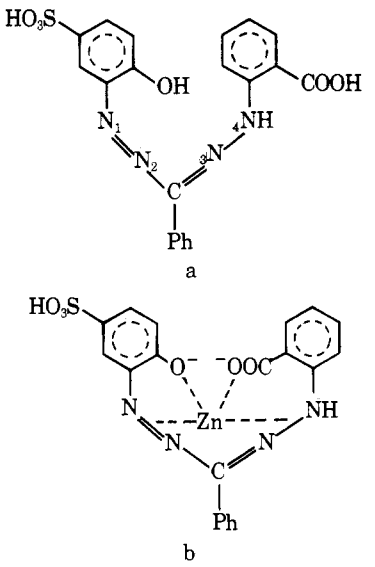

FIGURE 1.

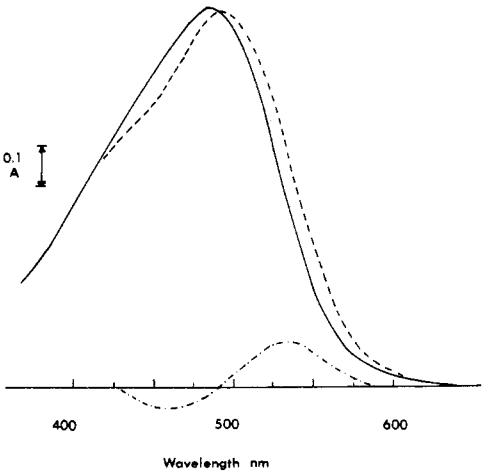

FIGURE 2: Visible-ultraviolet spectra of zincon and zincon-enzyme complex in pH 8.75, 0.05 M pyrophosphate. [Zincon] = 4.5×10^{-5} M; [enzyme] = 7.1×10^{-5} M. (—) Spectrum of zincon; (— — —) spectrum of zincon-enzyme complex; (— · —) difference spectrum of zincon-enzyme complex.

Experimental Section

Reagents. The enzyme preparation has been described previously (Bernhard *et al.*, 1970). Zincon was reagent grade, obtained from Fisher Scientific and used without further purification. Dithizone was Matheson Coleman and Bell reagent grade also used without further purification. Zinc nitrate was reagent grade from Baker Chemical. D_2O (99.5%) was obtained from Aldrich Chemical.

Kinetic and Spectral Experiments. Visible spectra of zincon in pH 8.75, 0.05 M pyrophosphate buffer, pH 2.5, 0.05 M citrate buffer, and pH 7.0, 0.05 M phosphate buffer, as well as the zincon-enzyme complex in pH 8.75, 0.05 M pyrophosphate solution and zinc-zincon in pH 8.75 unbuffered solution are shown in Figures 2 and 3. These spectra were recorded on a double beam Beckman Acta V spectrophotometer. The difference spectrum of the enzyme-zincon complex was obtained by forming the complex in the sample cell and placing zincon in the reference. The following conditions obtained in this experiment: [zincon] = 4.5×10^{-5} M; [enzyme] = 7.1×10^{-5} N; approximately 70% of zincon is complexed with enzyme under these conditions. The enzyme-zincon difference spectrum disappears upon formation of enzyme-NAD^+-pyrazole ternary complex at both pH 8.75 and pH 6.5; concentrations of NAD^+ and pyrazole were 1×10^{-4} and 1×10^{-2} M, respectively.

Liver alcohol dehydrogenase, 3.5×10^{-5} N, was titrated with zincon in pH 8.75 pyrophosphate buffer; zincon was added in increments to both sample and reference cell. The resulting graph of $\Delta OD_{535\,nm}$ against zincon concentration showed hyperbolic behavior. Standard iterative computer techniques were employed to calculate the binding constant, extinction coefficient, and the number of independent binding sites.

Steady-state kinetics were carried out under the following conditions: [enzyme] = 5.6×10^{-9} N, [benzaldehyde] = 1.6×10^{-4} M, and [NADH] = 6×10^{-6}–6×10^{-5} M at the following zincon concentrations: 0, 2.1×10^{-5}, 4.2×10^{-5}, and 8.4×10^{-5} M. A series of straight lines with a common y-axis intercept indicating linear competitive inhibition by zincon was obtained from plots of 1/velocity against 1/[NADH]. Because of the intense absorption of zincon at 340 nm, quantitative interpretation of experimental data collected at high zincon concentrations is difficult. Although inhibition is clearly competitive, values of inhibition constants are not precise as noted. K_I was estimated to be 0.5–2.0 $\times 10^{-5}$ M; all kinetic data were subjected to linear regression analysis.

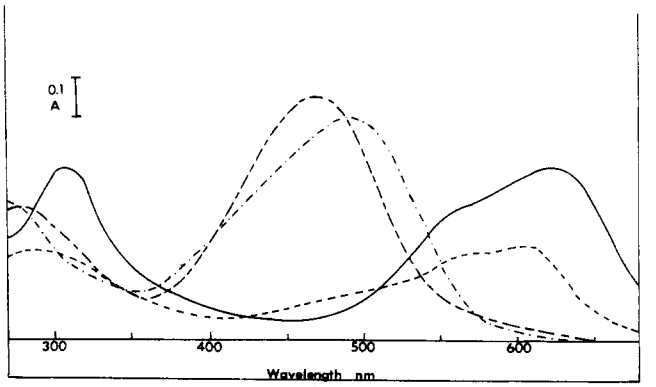

FIGURE 3: Visible-ultraviolet spectra of zincon and zincon-zinc complex. [Zincon] = 2.8×10^{-5} M. (— · —) Spectrum of zincon in pH 8.75, 0.05 M pyrophosphate; (— · — · —) spectrum of zincon in pH 7.0, 0.05 M phosphate; (— — —) spectrum of zincon in pH 2.5, 0.05 M citrate; (—) spectrum of zincon-zinc complex in pH 8.9, unbuffered.

Laser-Raman Experiments. Samples were prepared under buffer conditions listed above. Zincon concentration was measured using ϵ 21,000; λ_{max} 480 nm at pH 8.75. Solutions were prepared at concentrations shown in spectral figures by mixing stock solutions and putting them into capillary tubes for Raman spectral analysis. The zinc-dithizone complex was extracted from water with CCl_4; only the neutral complex is soluble in the organic layer.

Raman spectra were recorded in melting point capillaries using the Spex 1401 laser Raman spectrometer with the Coherent Radiation Laboratories Model 52 argon ion laser as the source. All spectra were recorded using both the 514- and the 488-nm laser wavelengths for excitation. In some cases the 514-nm line produced the superior spectrum while in others the situation was reversed. In each sample the observed Raman shift frequencies were independent of excitation frequency. Spectral slit widths were ≤ 5 cm^{-1} in all cases, and accuracy was at least ± 5 cm^{-1}. No effort was

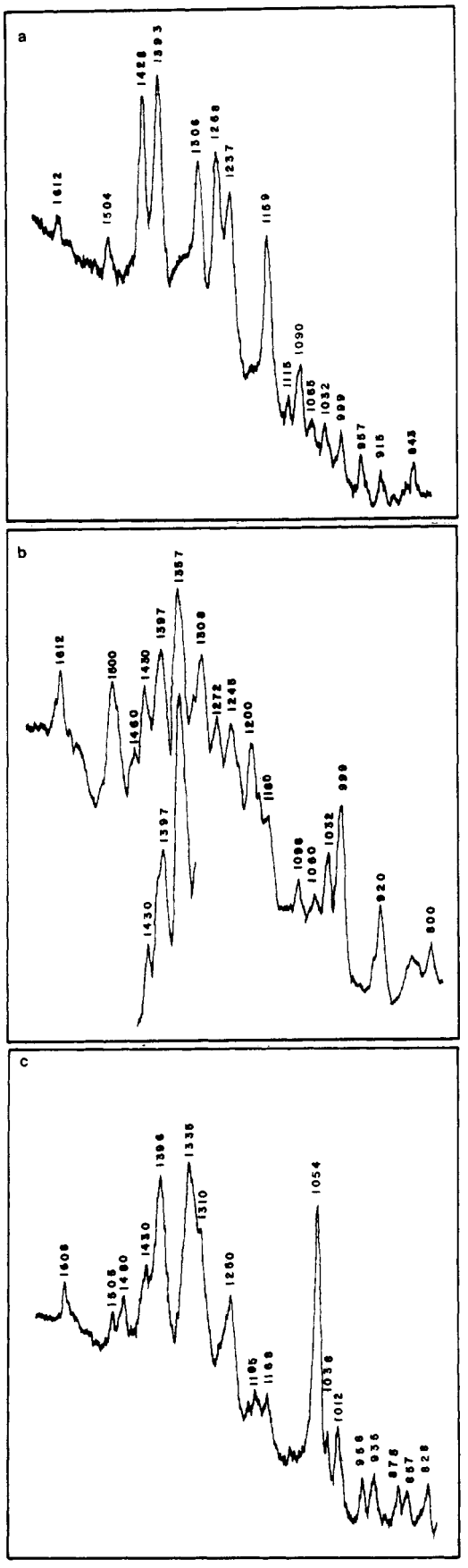

FIGURE 4: Resonance Raman spectra of zincon, zincon-enzyme complex, and zincon-zinc complex. Solutions are buffered at pH 8.75 by 0.05 M pyrophosphate (a) Spectrum of zincon, [zincon] = 5.0×10^{-5} M; (b) spectrum of zincon-enzyme complex, [zincon] = 5.0×10^{-5} M, [enzyme] = 7.1×10^{-5} M; insert [zincon] = 5.0×10^{-5} M, [enzyme] = 1.26×10^{-4} M; (c) spectrum of zincon-zinc complex [zincon-zinc] = 2×10^{-4} M. Solution is unbuffered.

made to determine depolarization ratios for these spectra. Spectra did not vary with time of irradiation.

The Raman spectrum characteristic of the pH 8.75 enzyme-zincon complex completely disappears on formation of the NAD^+-pyrazole-enzyme ternary complex under the following concentration conditions: [enzyme] = 7.1×10^{-5} N, [zincon] = 5.6×10^{-5} M, [NAD^+] = 5×10^{-3} M, [pyrazole] = 5×10^{-3} M. Correspondingly there is no intensity change in the Raman spectrum on formation of enzyme-NAD^+-pyrazole complex at pH 6.5 under the following concentration conditions: [enzyme] = 3.5×10^{-5} N, [zincon] = 2.9×10^{-5} M, [NAD^+] = 2.0×10^{-4} M, [pyrazole] = 2×10^{-2} M. Relative intensity values were determined using vibrational bands of buffer as an internal standard.

Results and Discussion

Discussion of Spectral Observations. The visible absorption spectrum of a complex between liver alcohol dehydrogenase and zincon is shown in Figure 2. Spectral titration of liver alcohol dehydrogenase with zincon shows normal hyperbolic behavior and gives an isosbestic point; the titration data are consistent with a binding constant, K_{eq}, of 1.6×10^{-5} M ($\Delta\epsilon$ = 3600 l. M^{-1} cm^{-1}) and n (the number of independent binding sites per enzyme monomer) = 1. Experiments in which zincon is studied as an inhibitor of the enzyme-catalyzed reduction of benzaldehyde by NADH indicate that zincon is a strictly competitive inhibitor of NADH binding with $K_I = 0.5\text{-}2.0 \times 10^{-5}$ M. The visible spectra of zincon at pH 2.5 and pH 7.0 as well as the spectrum of the 1:1 zinc complex of zincon are shown in Figure 3.

Since both zincon and the zincon-enzyme complex have large extinction coefficients at both 514 and 488 nm, the laser wavelengths used for Raman excitation with our Raman spectrometer, both were potential resonance Raman scatterers. Figures 4a and b show the resonance Raman spectra obtained for zincon and for the zincon-enzyme complex, respectively (70% of zincon present is bound to enzyme). For comparison, Figure 4b also presents a second point on the titration curve in which 85% of zincon is enzyme bound. Since at these concentrations the enzyme gives no Raman spectrum, the differences between Figure 4a and b must reflect the effects of enzyme complexation upon zincon. These differences include the appearance of a new, intense band at 1357 cm^{-1} and a weaker band at 1200 cm^{-1}.

Even though the electronic absorption band for the zinc-zincon complex shifts far toward the red end of the spectrum and away from the Raman excitation frequency, we have obtained a resonance Raman spectrum of the zinc-zincon complex at a concentration of 2×10^{-4} M (Figure 4c). Comparison of zinc-zincon complex and the enzyme-zincon complex shows a great deal of spectral similarity between 1650 and 1100 cm^{-1}. These similarities include the appearance of two bands which are not present in zincon: (1) 1357 cm^{-1}, enzyme-zincon; 1335 cm^{-1}, zinc-zincon; and (2) 1200 cm^{-1}, enzyme-zincon; 1195 cm^{-1}, zinc-zincon. Table I catalogs the spectra of zincon, enzyme-zincon, and zinc-zincon for comparison.

Previous studies of azo dyes indicate that stretching vibrations of the N=N and C=N bonds occur in the 1380-1650-cm^{-1} region (Hacker, 1965; Goulden, 1953). We have also studied the resonance Raman spectra of zinc and nickel complexes of another formazyl dye (dithizone); these complexes show a new, very intense band at 1347 and 1333 cm^{-1}, respectively, which are not present in the free ligand. Previous ir studies of azo dye complexes such as the Cu and

Table I[a]

Zincon	Zincon–Enzyme	Zincon–Zinc
1612	1612	1608
1504	1500	1505
	1460	1480
1428		
1393	1397	1396
	1357	1335
1306	1308	1310
1268	1272	
1237	1245	1250
	1200	1195
1159		
1115		
1090		
1065	1060	1054
1030[b]	1032[b]	1038[b]
999	999	1012
957	965	958
915	920	935
	880	878
843	840	857
	800	828
	620	605

[a] Frequencies in cm^{-1}. [b] This band results from pyrophosphate buffer.

Pd complexes of 1-(2-pyridylazo)-2-naphthol also show a new spectral band near 1350 cm^{-1} upon complex formation (Shibata, 1972). It is our conclusion that this new, intense band results from formation of a covalent bond between an azo group and metal.

There are also two significant differences between enzyme–zincon and zinc–zincon complexes in this region, namely the 1500-cm^{-1} band which is very intense in the enzyme complex but is weak in zinc–zincon and the 1272-cm^{-1} band in the enzyme complex which is not present in zinc–zincon. The spectral region below 1100 cm^{-1} shows differences between enzyme–zincon and zinc–zincon complexes; these differences will be dealt with below.

Despite the Raman spectral similarities between enzyme–zincon and zinc–zincon complexes, it is clear from the visible spectral shifts that these complexes are not identical (Figures 2 and 3). We have studied pH dependence of Raman spectra of zincon and the spectrum of zincon in D_2O to determine which spectral bands are affected by changes at various ionizable groups. Although we make no specific spectral assignments, we have assumed that bands which change upon protonation or deuteration of ionizable groups (*e.g.*, the carboxylic acid or phenol groups in zincon) would also change upon zinc complexation. Table II lists those bands in the Raman spectrum of zincon which change as a result of (1) protonation of the phenol group (pK_a = 8.3, compare columns a and b), (2) protonation of carboxylate (pK_a = 4.5, compare columns a and c), and (3) substitution of deuteron for proton on the formazyl nitrogen (N-4 in Figure 1a) (compare columns a and d). There are no other ionizable groups in zincon with pK_a's between 3.0 and 10.0. This has been confirmed by our studies on formazans having no carboxyl or phenol groups. We find no pH dependence of visible or Raman spectra between pH 3.0 and pH 10.0 for these species.

Table II[a]

pH 8.75	pH 6.2	pH 3.0	pD 8.75 (D_2O)
1612	1610	1610	1612
	1590		
1504	1500	1507	1485
1428			1430
1393	1394	1400	1396
1306	1305	1318	1305
1268			1270
1237	1228	1231	1245
1159	1177	1180	1160
1115			
1090	1095	1090	1090
1065			
999	998	1000	
957	952	954	
915	910	915	
			870
			840

[a] Frequencies in cm^{-1}.

A comparison of the zincon spectra at pH 8.75, 6.2, and 3.0 shows that there are very minor differences between resonance Raman spectra at pH 6.2 and 3.0 while the pH 6.2 spectrum differs considerably from the pH 8.75 spectrum. Bands at 1159, 1268, and 1428 cm^{-1} in pH 8.75 zincon either shift or disappear on protonation of phenol. These bands also shift or disappear upon formation of the zinc–zincon complex in which the phenol group is complexed by zinc. Therefore, we interpret similar shifts of these bands in the enzyme complex as indication that the phenol group is either protonated or bound to a zinc atom on the enzyme surface. (The decreasing relative intensity of the 1430- and 1160-cm^{-1} bands as the percentage of free zincon decreases in Figure 4b indicates that these bands are due only to free zincon. This is also true for zinc–zincon in Figure 4c in which a slight excess of free zincon is necessary to keep zinc in solution at pH 8.75; spectra of zinc–zincon with excess zinc in highly buffered systems show this band to be absent.) We have not explained the fact that the 1268-cm^{-1} band in zincon is unshifted upon enzyme complex formation even though this band does shift when zincon complexes free zinc.

The absence of significant Raman spectral changes between pH 6.2 and 3.0 indicates that the resonance Raman spectrum does not provide information about the chemical environment of the carboxylate group. However, the only large pH-induced change in the *visible* spectrum of zincon results from protonation of carboxylate. A similar large red shift occurs upon complexation of the carboxylate in the zinc–zincon complex. The absence of such a shift in the enzyme complex provides evidence that the zincon is not complexed to the enzyme surface through the carboxylate.

Finally, information on the formazyl involvement in enzyme complexation of the zincon can be gained by studying the spectrum of zincon in D_2O at pD 8.75. At this pD, the phenol and carboxylate groups are unprotonated, leaving the formazyl hydrogen as the only one readily replaced by deuterium. Therefore, the spectral shifts observed should be due to vibrations involving motions in the formazyl nitrogens. The bands which shift upon deuterium substitution

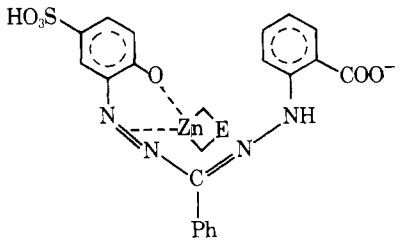

FIGURE 5.

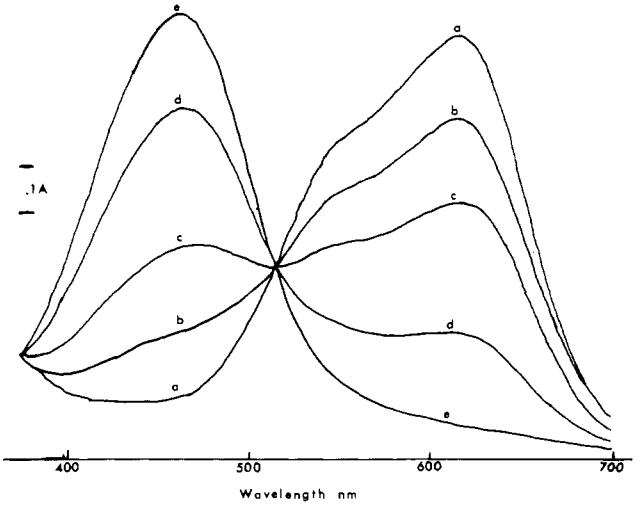

FIGURE 6: Proton competition experiment with zinc-zincon. [zinc] = 7.0×10^{-5} M; [zincon] = 4.7×10^{-5} M. (a) pH 8.2; (b) pH 7.2; (c) pH 6.9; (d) pH 6.5; (e) pH 6.1.

are those between 900 and 1050 cm^{-1} which shift to the 800–900-cm^{-1} region (Table II) and the band at 1504 cm^{-1} which shifts to 1485 cm^{-1}. The formation of the zinc-zincon complex also produces Raman spectral changes in these regions pointing to formazyl complexation as expected. However, with the exception of intensity changes, the enzyme complex spectrum looks very much like free zincon in these regions leading us to conclude that the formazyl nitrogens of zincon are not complexed at the enzyme surface.

In summary, we have observed the resonance Raman spectrum of zincon bound to liver alcohol dehydrogenase. The spectrum of the enzyme complex differs considerably from free zincon; these differences are much more extensive than those generated on binding a dye (Methyl Orange) noncovalently to bovine serum albumin (Carey *et al.*, 1972). This suggests that the large number of changes in our zincon–liver alcohol dehydrogenase spectrum might result from covalent interactions with metal atoms. Specifically the appearance of a new band in that Raman region assigned to N=N stretch in azo dyes seems to be diagnostic of formation of a covalent bond between metal and the nitrogen ligand. This new band is *only* observed upon metal complexation; the change could result either from direct effects upon the N=N band or from conformational change induced by complex formation. The enzyme complex also shows a number of changes in that region of the spectrum which is significantly affected by protonation of the phenol group and by zinc complexation of the same phenol group in zinc–zincon. This leads us to believe that the phenol is either complexed to zinc or protonated on the enzyme surface. Differences between zinc–zincon and enzyme–zincon complexes are observed for Raman spectral bands which change upon deuteration; since the only labile hydrogen which is not ionized at pH 8.75 resides on N-4 of the formazyl system (Figure 1a), we interpret our data to indicate that this nitrogen is *not* involved in complex formation with zinc in the enzyme complex. Likewise, the visible spectra suggest that the carboxylate is not complexed to zinc in the enzyme complex; the large red shift which occurs on complexation of carboxylate in zinc–zincon and on protonation of the carboxylate is not observed in the zinc–enzyme complex. Figure 5 illustrates our proposed model, indicating covalent binding between zincon and a zinc on the enzyme surface. We have indicated that the phenol is covalently bound to zinc; however, another possibility is that it is protonated rather than coordinated to zinc. Even though our interpretation of the data leads us to postulate direct complexation to zinc, this complexation is obviously quite different from that found in the 1:1 zinc–zincon complex. We

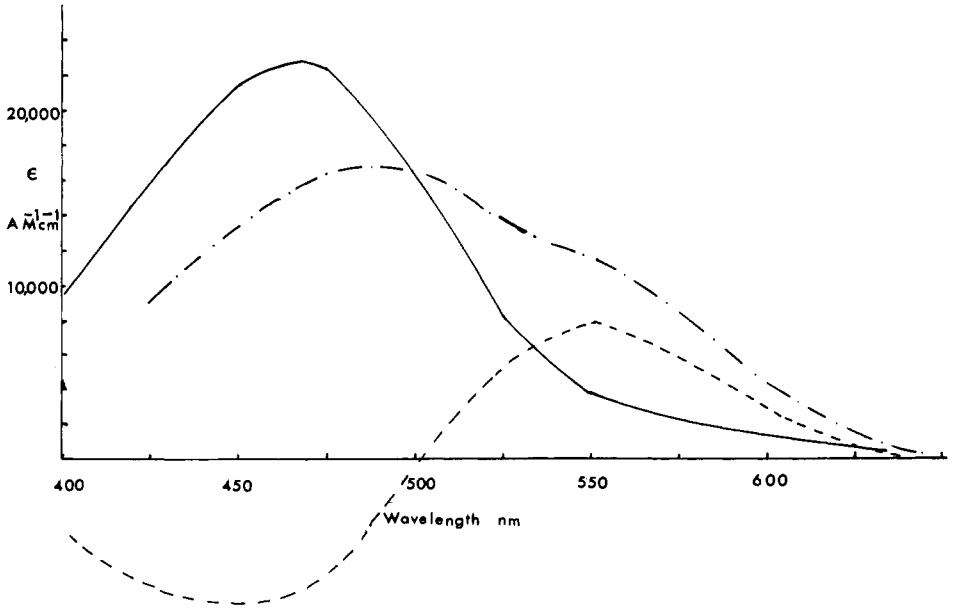

FIGURE 7: Visible spectra of pH 6.5 zincon-enzyme complex. (—) Spectrum of zincon, pH 6.5; (— · —) spectrum of zincon-enzyme complex; (— — —) difference spectrum of zincon-enzyme complex.

therefore can offer no chemical model with zinc-ligand bonding corresponding to our proposed enzyme complex, and cannot at this time rule out other explanations which do not invoke direct ligand-metal interactions.

pH Dependence of Enzyme Complexation by Zincon. In order to further test our hypothesis that zincon is bound to the active site zinc atom at pH 8.75, proton competition experiments have been performed. Zinc-zincon undergoes increasing dissociation to free zinc and free ligand as the pH of a solution containing zinc-zincon is decreased (Figure 6). The concentration of zinc and zincon in this experiment is approximately equal to the concentration of enzyme and zincon, respectively, in the enzyme complexation experiment. Similar proton competition experiments with the enzyme-zincon complex produce a new complex as the pH is lowered. Figure 7 shows the electronic spectrum of this complex at pH 6.5; the spectrum is clearly different from the basic complex showing a much larger red shift of the zincon spectrum on enzyme binding. Spectral shifts of this magnitude have been previously shown to result from noncovalent binding to the hydrophobic binding site (Bernhard *et al.*, 1966). Further support for the interpretation is the similarity of the spectrum of the pH 6.5 enzyme-zincon complex and the spectrum of zincon in dixoane (a solvent of low dielectric constant). The dioxane solution of zincon shows a red shift in the electronic spectrum to λ_{max} 532 nm. Titration of enzyme with zincon at pH 6.5 yields a good isosbestic point indicating a single enzyme-zincon complex; the binding constant for the complex is $K_{eq} = 2.7 \times 10^{-5}$ for $n = 1$. Steady-state kinetic experiments indicate that the site of zincon binding overlaps the NADH binding site since strictly competitive inhibition ($K_I = 0.8\text{-}1.5 \times 10^{-5}$) is observed.

The resonance Raman spectrum is strikingly different from that of the basic complex (Figure 8); at pH 6.5 the vibrational spectrum of enzyme bound zincon is nearly identical with the spectrum of free zincon in spite of the fact that the visible spectral changes indicate that zincon is tightly complexed to enzyme. The absence of Raman spectral change upon complex formation at pH 6.5 could suggest that a weak Raman spectrum for the bound ligand is obscured by a strong spectrum from the unbound ligand. If this were the case, we would expect an intensity increase upon displacement of zincon from the enzyme complex. However, the absence of Raman intensity changes upon displacement of zincon from the enzyme active site by NAD^+-pyrazole argues that we are observing zincon complexed to enzyme as well as uncomplexed zincon. Again this is consistent with noncovalent hydrophobic binding between enzyme and zincon at pH 6.5.

Conversely the observation of the large vibrational spectral shift in the basic enzyme complex indicates that these do not result from simple noncovalent binding to a hydrophobic enzyme site. The similarity between displacement of zinc by protons in the zinc-zincon complex and the change with pH in the vibrational spectrum of the enzyme complex indicates that both phenomena are the result of the breaking of metal-ligand bonds due to displacement by protons.

A large pH dependent conformational change of the enzyme might also explain the changes in the vibrational spectrum with pH; such changes might result if zincon were conformationally deformed by binding to the enzyme site at pH 8.75. However, we are aware of no evidence suggesting such a large pH dependent isomerization of the enzyme.

Molecular Interpretation. Since zincon is a competitive

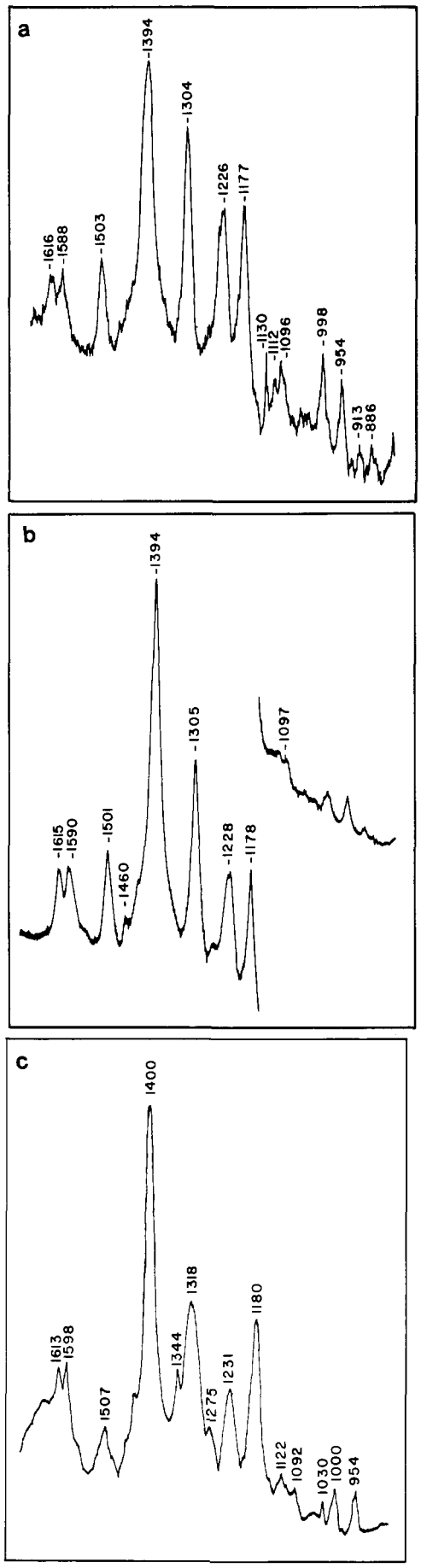

FIGURE 8: Laser Raman spectra at pH 6.5 and pH 3.0. (a) Free zincon, pH 6.5; [zincon] = 8×10^{-4} M; (b) zincon-enzyme complex, pH 6.5; [zincon] = 4×10^{-5} M; [enzyme] = 9×10^{-5} M; (c) free zincon, pH 3.0; [zincon] = 1×10^{-4} M.

inhibitor of coenzyme binding, our data indicate that zincon is directly complexed to zinc at the active site. This is in accord with recent X-ray data which indicate the presence of a zinc atom near the coenzyme binding site (Brändén *et al.*, 1973), and is consistent with similar locations for this zinc atom in the crystal and in solution. There are two zinc atoms present per monomer of enzyme—one an easily dialyzable "catalytic" zinc atom necessary for catalytic activity and a second "structural" zinc atom (Drum and Vallee, 1969). Since zincon binds in a strictly competitive fashion to the coenzyme binding site, that zinc atom forming the covalent complex with zincon is the catalytic zinc atom. The observation that a NAD^+-pyrazole complex completely displaces zincon from its binary complex with the enzyme (as indicated in both visible and Raman spectra) supports the conclusion that there is no mixed complex formed in which the zincon is bound to both "structural" and "catalytic" zinc. In conclusion, the simplest hypothesis at this time seems to be that zincon is bound at the "active site" or catalytic zinc at either one or two coordination sites on that zinc.

Several interesting conclusions can be drawn from the observation of both noncovalent and covalent complexes between enzyme and zincon. Most of the binding free energy is supplied by noncovalent hydrophobic interaction in both the neutral and basic complexes. The binding constants at pH 6.5 and 8.75 indicate only a slight tightening of complexation as the result of covalent bond formation to zinc at the active site. While this observation is unexpected in view of our proposal that a ligand–metal bond is formed at pH 8.75 but not at pH 6.5, other studies have reported similar binding constants for both metal chelating (bipyridyl, *o*-phenanthroline [Sigman, 1967]) and nonchelating (Rose-Bengal and 8-anilino-1-naphthalenesulfate [Brand *et al.*, 1967]) reagents. Even though one of these, *o*-phenanthroline, is clearly complexed to zinc at the enzyme active site (Brändén, 1973), its binding free energy is not appreciably different from that for Rose-Bengal binding to liver alcohol dehydrogenase. One might expect that binding of substrate and metal at the active site of a metalloenzyme might have significant catalytic importance without having any great affect on the free energy of binding of substrate.

The data also indicate that the absence of large electronic spectral changes upon complexation of a chromophore to an enzyme active site does not preclude covalent interactions in the complex. The spectrum of the basic zincon–enzyme complex is blue shifted toward the electronic transition for free zincon. The net result of the red shift due to hydrophobic interaction and the blue shift due to metal–ligand binding is that the spectrum of the complex is only very slightly shifted from that of free zincon. This indicates that the resonance Raman technique is a very valuable tool especially for studying metalloenzymes.

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