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Cobaltous Ion Complex of Reduced Lipoamide Dehydrogenase†

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ABSTRACT: Lipoamide dehydrogenase is a dimeric flavoenzyme with no metal requirement. Nicotinamide adenine dinucleotide reduced lipoamide dehydrogenase under anaerobic conditions at pH 10 complexes with Co(II) generating a unique absorption band at 650 nm with molar absorptivity of 980 based on flavine adenine dinucleotide (FAD^+) concentration. Optically active bands appear at 465 and 395 nm. Complex formation is stoichiometric with respect to FAD^+ and has a $pK = 8.25$. The $Co(II)_2$LipDH complex, when returned to pH 7, is stable in air with minimal native reductase activity. 1,10-Phenanthroline reverses cobalt binding at higher pH. Of the common divalent metals only cobalt exhibits these properties. This stable and specific complex appears to be the first demonstration of its kind, based on chemical reactivity. The cobalt-binding site of this enzyme is probably distinct from the arsenite- and cadmium-sensitive sites, because the diaphorase activities of the modified enzymes are quite different, and since complexation properties of the NADH-reduced enzyme are quite different from those of the dihydrolipoate-reduced enzyme, the equilibrium states of the two reduced enzymes are not the same.

The spectroscopic usefulness of cobaltous ion as a structural probe for enzymes has been documented by Latt and Vallee (1969) for carboxypeptidase, by Simpson and Vallee (1968) and Applebury and Coleman (1969) for alkaline phosphatase, by Lindskog and Nyman (1964) for carbonic anhydrase, and by Drum and Vallee (1970) for liver alcohol dehydrogenase. All these enzymes possess a site tailored to metals and require no other cofactors. As far as we know, there is no published attempt to induce a nonmetalloflavoenzyme to bind cobaltous ions uniquely and specifically and to investigate the resultant complex as a probe of active-site structure. The principal reason is that nonmetalloproteins are not expected to bind metals in a uniquely liganded fashion. Certain proteins are known to bind a spectrum of metals nonspecifically, one example being casein (Minato and Tanaka, 1955). These do not generate colored complexes and may simply be the result of salt linkages.

The affinity of cobaltous ion for amino acids, especially the imidazole group of histidine and dipeptides, is well established (Morris and Martin, 1970). Colored tetrahedral complexes form in basic solution anaerobically after deprotonization of the liganding imino nitrogen. Free flavines, particularly the semiquinone form, also possess metal binding capacity (Hemmerich *et al.*, 1965). We therefore sought to use cobalt as a structural probe for the flavoprotein lipoamide dehydrogenase. As with a few other transition metals having unfilled d shells, cobalt (d^7) possesses the ability to generate colored complexes, wherein the d–d transitions become susceptible to spectral analysis. Under appropriate conditions, cobalt can be expected to interact with either the semiquinone flavine or vicinal amino acid residues or both. The nature of the complex may be interpretable on the basis of either one of the above models. Formation of such a flavoprotein–metal complex may also shed light on the nature of metal binding in metalloflavoproteins. At this time, only iron and molybdenum are known to be essential in any metalloflavoenzyme, and the strong affinity of the free semiquinone flavine for metals is apparently lost once it is bound to a nonmetalloflavoprotein. Beinert and Hemmerich (1965) have used the loss of metal affinity for protein-bound semiquinone to suggest that the free semiquinone–metal complex is probably of no significance in metal binding in metalloflavoenzymes. They have given evidence based on epr studies for the forced interaction of Fe(III) and Ni(II) with glucose oxidase, but only with a 100-fold excess of paramagnetic ion.

We wish to present here evidence for the reaction of pig heart lipoamide dehydrogenase, a nonmetalloflavoprotein, with cobalt to form a specific complex with unique absorption and circular dichroic spectra.

Materials and Methods

Pig heart lipoamide dehydrogenase, type III, was obtained from Sigma Chemical Co., St. Louis, Mo. Beef heart lipo-

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amide dehydrogenase from Miles-Seravac, Maidenhead, England, was found to give the same results. All the data presented here, however, were obtained with the Sigma enzyme. *Neurospora crassa* NADase,[1] obtained from Sigma Chemical Co., was assayed according to Colowick *et al.* (1951a,b). Solubilized enzyme was used within 1 day. For treatment of the Co–LipDH complex, total NADase activity was 240 mU in 1.7 ml at pH 7.5, 25°. Flavine concentration was 40 μM and the NADase is not inhibited by cobalt, LipDH, or the Co–LipDH complex.

Determinations of purity of the purchased enzyme were made by polyacrylamide gel electrophoresis at pH 8.3 in 40% sucrose. Only a trace amount of protein other than the native enzyme was found, and that band had the same mobility as a freshly prepared solution of apolipoyl dehydrogenase made by a previously described method (Brady and Beychok, 1969).

Enzyme concentration was determined on the basis of A_{455} and that value assigned an E^{M}_{455} of 11,300 (Massey *et al.*, 1962). Lipoate reductase activity was determined according to Massey *et al.* (1960) with 50 mM K_2HPO_4 buffer and 30 μM EDTA. Transhydrogenase activity was determined according to Ide *et al.* (1967) with the thionicotinamide adenine dinucleotide analog of NAD^+[1] (Sigma Chemical Co.) in 100 mM K_2HPO_4 at pH 7.5. Specific diaphorase potential was obtained according to Casola *et al.* (1966) with 2,6-dichlorobenzenoneindophenol (DCIP).

Stable enzyme at pH 10 was obtained by dialyzing the enzyme against 50 mM borate buffer at pH 10. By this means, the pH 10 enzyme was indistinguishable from the native enzyme at pH 7 with respect to specific activity (determined at pH 5.65), absorption, and circular dichroic (CD) spectra. At lower pH, 50 mM phosphate buffer was used for all experiments reported here.

Anaerobic conditions were obtained by passing nitrogen into a plastic glove bag (Instruments for Research and Industry, Cheltenham, Pa.). The enzyme solution was placed in an evaporating dish over ice with occasional agitation. One–two hours was sufficient time for adequate deoxygenation as determined by persistence of the reduced red enzyme. Excess solid NADH (four- or fivefold) was added to obtain complete reduction to the semiquinone form of $FAD^{\cdot}$. A_{435}/A_{455} of 0.76 was taken as indication of complete reduction. Excess dihydrolipoic acid was added to obtain the corresponding reduced form of the enzyme.

Formation of the complex was performed by adding 10–15-μl amounts of a freshly prepared degassed solution of concentrated 10 mM cobalt sulfate to the enzyme in an anaerobic Thunberg cell. The red color of the half-reduced enzyme changed to bright yellow within a few seconds. The reaction is stoichiometric with respect to FAD^+ (Co:FAD^+ = 1:1). In most cases, a slight excess of cobalt was allowed (Co/FAD^+ = 1.27–1.03). Cobalt concentration was determined by the nitroso-R-salt procedure (Sandell, 1944).

Absorption spectra and enzyme activity measurements were obtained with a Cary 14 recording spectrophotometer. Optical circular dichroism was measured with a Cary 60 recording spectropolarimeter with CD attachment. The instrument was calibrated with *d*-camphorsulfonate at 290 nm. All spectral measurements were at ambient temperature (24°). Absorbance of samples was maintained in the range of 0.4–0.8 to maintain low dynode voltages (200–400 mV) on the spectropolarimeter.

Results

Lipoamide dehydrogenase complexes specifically with cobalt. Among the four other divalent metals, Mn(II), Fe(II), Ni(II), and Zn(II), only the last gave an unambiguous complex. Since zinc is the only noncolored ion in the series with a filled d shell, its reaction with the enzyme serves as a useful means of distinguishing bands with the flavine as chromophore from those originating from the metal. The zinc complex will therefore be discussed along with the Co complex. Co and Zn gave complexes at a 1:1 metal ion:FAD ratio. While the other metals may interact with the reduced enzyme (see below), they do not effect enzyme activity, are not stable in air, and do not yield new spectroscopically distinct species even at higher metal ion:FAD ratios.

Mn(II) was added to the enzyme in a molar ratio Mn:FAD = 340:24 at pH 10 under N_2. The visible absorption spectrum of the reduced enzyme at 430 nm was noted to increase gradually for about 35 min, until an intensity midway between that of the oxidized and the half-reduced enzyme was reached. Upon aeration of the complex, native absorption features returned with 75% of the initial lipoate reductase activity regenerated after 45 min of oxygen equilibration. The CD spectrum was essentially unchanged and no new bands were observed in the visible absorption spectrum out to 1300 nm.

The response of the half-reduced enzyme to Fe(II) ions was similar to that with Mn(II). With a Fe:FAD ratio of 54:40 and a 2-hr incubation at 25° there was complete transformation of the treated, reduced-enzyme spectrum to native, oxidized enzyme and subsequent aeration gave no further spectral changes. Lipoate reductase activity after 30 min of O_2 equilibrium returned to 75% of the native enzyme. The circular dichroism after dialysis of the complex back to pH 7 was identical with that of the native enzyme. The apparent oxygen-free reoxidation of the semiquinone by Fe(II) is puzzling because Fe(I) is not a stable redox state. If Fe(III) had been used a possible mechanism involving electron acceptance by the ferric ion from the semiquinone or mercaptide could be envisioned. As it is, a mechanism *via* the transient iron–NADH complex discovered by Gutman *et al.* (1968) is precluded since the metal is Fe(II). Even though Fe(II) does become air-oxidized to Fe(III) in strongly basic solution, such reactions involve the hydroxides and do not occur as rapidly as in the case here (<2 hr). Iron is an *in vivo* cofactor of certain metalloflavoproteins and numerous heme- and iron–sulfur proteins; however, lipoamide dehydrogenase has not been shown to possess a prestructured site for iron, and the effect of iron on this enzyme deserve further study.

The effect of Ni was determined at two different concentrations: Ni:FAD = 40:23 and 252:42. At the lower ratio nickel causes anaerobic reoxidation with the appearance of native enzyme absorption and CD spectra in 38 min. At this time lipoate reductase activity was 58% of the native enzyme. The higher Ni ratios did not affect the enzyme at all for 30 min at 25°. However, upon O_2 equilibration for 20 hr at pH 10 (4°), two bands appeared at 449 and 350 nm with the same intensity as that of free flavine. Lipoate reductase activity was down to 5% of the original. After dialysis the CD spectrum showed perturbations at the 355-, 333-, and 305-nm bands with $[\theta]_{365}$ = 18,700. There were no new transitions out to 520 nm. The different effects of Ni(II) with and without excess metal ions are not without precedent. Beinert and Hemmerich

[1] Abbreviations used are: NAD, nicotinamide adenine dinucleotide; NADH, reduced form; FAD^+, flavine adenine dinucleotide; LipDH, lipoamide dehydrogenase; DCIP, 2,6-dichlorobenzenoneindophenol.

TABLE I: Determination of E_{650} for Cobalt Enzyme.

Expt	FAD^+ Concn (μM)	Co/FAD^+	E^M_{650} (M^{-1} cm^{-1}) Anaerobic	O_2 Equilibrated
1	54	1.27	1170	820
2	60	1.03	1050	650
3	44	1.13	910	630
4	36	1.16	850	650
5	45	1.27	930	705
6[a]	41	0.78	940	
7[a]	42	0.48	1075	
8	33	1.84	1120	
9	44	1.09	955	745
10	40	1.20	1000	810
11	88	1.14		725
12	41	1.16	880	695

[a] The values for experiments 6 and 7 were based on the concentration of added cobalt. The other values were based on total FAD^+ concentration.

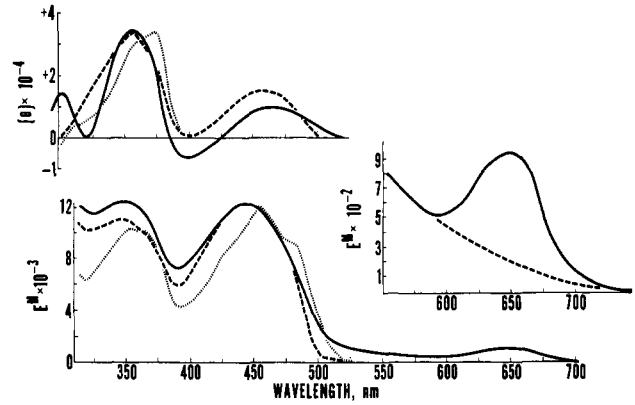


FIGURE 1: Absorbance and circular dichroism spectra of the cobalt (——) and zinc (- - - -) complexes. Both complexes were formed at equimolar concentrations of metal ion and enzyme flavine. The spectra of untreated lipoamide dehydrogenase are included for comparison (· · · ·). Insert shows E^M_{650} of the cobalt complex on expanded (10×) scale. The dashed line in the insert is the base-line correction. Both metal complexes were dialyzed in air at 4° for 20 hr against 50 mM K_2HPO_4 at pH 7. NAD^+ concentration is less than 1 μM. Molar ellipticity is in units of deg cm^2/dmol. All measurements were performed at 25°.

(1965) detected nickel–flavine interaction by increased spin relaxation in glucose oxidase, but only at a 100-fold excess of the metal. The effects of Mn(II), Fe(II), and Ni(II) are obviously quite distinct from those of Zn and Co. Only the latter two yield stable identifiable species. Furthermore, the failure to regenerate full lipoate reductase activity in the Mn-, Fe-, and Ni-treated enzymes can be attributed to the fact that at pH 10, where complexation is carried out, the enzyme is marginally stable and some denaturation is inevitable.

Figure 1 depicts the CD and absorption spectra of the cobalt and zinc complexes of lipoamide dehydrogenase. They are quite similar with the exception of additional bands in the cobalt complex. Table I lists the anaerobic absorptivities at 650 nm obtained on successive experiments of the cobalt complex, for which the average absorptivity is 980 M^{-1} cm^{-1}. This band is not optically active. For the zinc complex the 650-nm band is completely absent. The complex formation reaction is present and instantaneous, converting the red semiquinone to a bright yellow complex and generating bands at 350 and 450 nm.

In Table II are given the spectral characteristics of the cobalt and zinc complexes formed anaerobically at pH 10 with the NADH-reduced enzyme. The enzyme has been reduced with NADH in a 1:1 ratio; also, with a slight excess of NADH, the results have been the same. Stoichiometric amounts of manganous ions under these same conditions had no effect on the semiquinone spectra. Ferrous ions failed to produce the details given above, possibly because of anaerobic reoxidation of the semiquinone to oxidized enzyme. Equimolar concentrations of nickel gave absorption bands at 350 and 450 nm and 95% inhibition of enzyme activity after 20-hr incubation but still failed to generate A_{650} and $[\theta]_{395}$ or $[\theta]_{303}$.

A maximum of 30%, usually less, loss of oxygen-equilibrated A_{650} intensity can be achieved upon dialysis in phosphate buffer at pH 7. This is presumably from dissociation. Cobalt analysis showed essentially a 1:1 Co:FAD ratio. At pH 10, however, with 1.0 mM 1,10-phenanthroline dissociation of cobalt is substantially enhanced. The structured absorption band at 450 nm is restored, specific activity increased almost to that of the native enzyme, and all induced CD and the A_{650} bands are eradicated.

The dihydrolipoate-reduced enzyme does not form the cobalt complex described above. This finding corroborates with the results of Brady and Beychok (1971) indicating that the environment of the flavin in the NADH- and dihydrolipoate-reduced states are not equivalent, as shown by differing CD spectra. The absence of the A_{650} band complex in the cobalt-treated, dihydrolipoate-reduced enzyme suggests structural differences in the semiquinone–mercaptide diradical. Unlike the NADH-reduced enzyme, the dehydrolipoate-reduced enzyme is unstable at pH 10. After aeration flavine is released, and the protein precipitates when dialyzed with buffer at pH 7. The cobalt-treated enzyme, however, remains in solution when dialyzed to pH 7. Equimolar concentrations of metal ion to enzyme flavine were also used in these studies.

TABLE II: Spectral Parameters of the Cobalt and Zinc Complexes.

	350 nm	440 nm	650 nm
Anaerobic Molar Absorptivities (M^{-1} cm^{-1})			
Cobalt complex	*a*	11,000 (438 nm)	980
Zinc complex		12,000	Absent
Aerobic Molar Absorptivities			
Cobalt complex	11,000[b]	12,000	700–800
Zinc complex	11,000[b]	12,000	Absent

	303	355	395	465
Aerobic Molar Ellipticities (deg cm^2/dmol)				
Cobalt complex	17,000	35,000	−6000	9,000
Zinc complex	Absent	35,000	Absent	11,000

[a] Region dominated by excess NADH. [b] Approximate values, since no correction was made for residual protein absorption in this region. Both complexes formed by reaction of equimolar concentrations of metal ion and enzyme flavine.

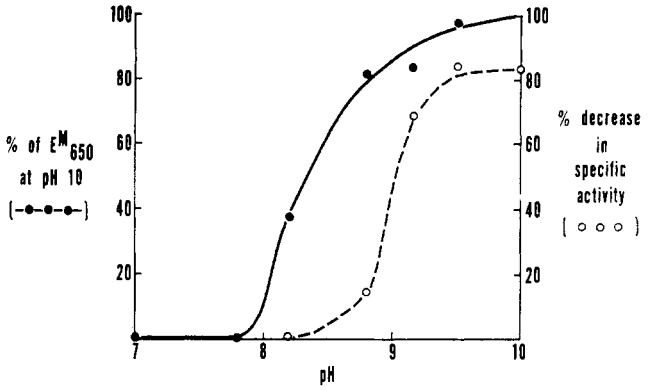


FIGURE 2: pH titration curve for cobalt complex formation. Specific activity is for lipoate reductase. The left ordinate, percent of E^M_{650} at pH 10, refers to the ratio of the E^M at each pH to the average E^M obtained at pH 10. Table I tabulates the determinations with an approximately 1:1 ratio of Co:FAD$^+$. The same ratio was used at each pH value in the figure above. The right-hand ordinate, percent decrease in specific activity, refers to the loss of specific activity relative to the untreated enzyme. Specific activities were obtained 2–3 hr after aeration of the complex, when E^M_{650} reached a minimum.

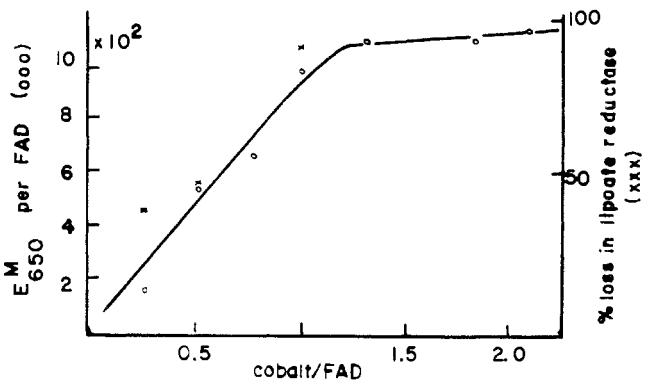


FIGURE 3: Cobalt titration curve for cobalt enzyme. Specific activity is for lipoate reductase. The left ordinate refers to the E^M_{650} at each value of [Co]/[FAD$^+$] to that at [Co]/[FAD] = 1 obtained from the average in Table I. Enzyme concentrations are based on FAD$^+$ absorptivity before cobalt is added. The right ordinate is identical with that in Figure 2.

One explanation of the different affinity of cobalt for the NADH- and dihydrolipoate-reduced enzymes might have been that Co complexes directly with enzyme-bound NAD$^+$. This appears unlikely. In the native enzyme 1 mol of NAD$^+$ is bound per flavine reduced, and upon aeration the NAD$^+$ is released. To verify in the cobalt-treated enzyme that no NAD$^+$ remained bound after aeration, sodium cyanide was added in 2000-fold excess. No 325-nm band arose characteristic of the NAD$^+$–CN$^-$ complex (Colowick *et al.*, 1951a,b). In addition, treatment of the complex with *Neurospora crassa* NADase at pH 7 for 7 min (37°) had no effect on the A_{650} band. The experiment was also performed by incubating the cobalt complex at pH 7.5 and 25° for 2 hr with NADase, without reduction in A_{650}. An aliquot of the mixture was then assayed for NADase activity at the end of the period and retained full NADase activity. Since NADase is inactive at pH 10, its effects on the complex could not be studied in basic solution.

Figure 2 shows the pH dependence curve for the formation of the complex of cobalt. Each point represents an individual experiment and a separate enzyme preparation. We have done this to avoid complications arising from precipitation of cobalt in basic solution and from sequential pH adjustment, which usually causes a small amount of turbidity from protein denaturation with resultant decreases in specific activity, and to avoid the errors of too much NAD$^+$ or NADH in the system. Figure 2 also plots the fractional decreases in lipoate reductase specific activity as a function of pH. It is interesting that the two curves do not overlap. The pK for complex formation is 8.25.

In the 200–250-nm region the CD spectra of these three proteins are substantially unchanged from that of the native enzyme. There is, furthermore, no difference in the ratio of the 222-nm minimum to that of the 207-nm minimum in the three materials. We take this ratio to reflect the constancy of helix content between them. Sedimentation analysis was carried out on the native, Co-, and the phenanthroline-treated Co-enzyme, demonstrating the identity of the dimer form of each material.

The experiments of Figure 2 also are proof that the spectral changes seen in absorption and circular dichroism of Co–lipoamide dehydrogenase are not simply pH-dependent alterations, because each point on the Co complex titration curve (solid dots) has essentially the same E^M_{650} when returned to pH 7 as it had at its respective pH on the curve. We qualify this last statement with the word "essentially" because the E^M_{650} of all samples decrease by at least 10% upon return to pH 7. This loss corresponds to a denaturation and release of a small amount of FAD$^+$ from the protein.

The cobalt titration curve is shown in Figure 3. Again, each point is a separate enzyme preparation. A generally linear relationship is obtained for increasing amounts of cobalt until a 1:1 ratio to FAD$^+$ is obtained. The fractional decrease in lipoate reductase is also plotted. The near linearity of the cobalt titration plot suggests lack of cooperative effect in the binding of the cobalt near each FAD$^+$ and identity of the two sites.

Figure 4 depicts the near-ultraviolet CD (300–250 nm) of the native enzyme Co^{II}_2LipDH and the cobalt–protein after dialysis against *o*-phenanthroline. The CD spectrum of Zn_2-LipDH in this region is almost identical with that of the cobalt complex.

Discussion

Lipoamide dehydrogenase easily lends itself to spectroscopic study. From the many investigations too numerous to cite here this enzyme has been shown to exhibit particularly intense flavine fluorescence and optical activity. It is perhaps as much for these peculiarities as out of interest in its protein structure that lipoamide dehydrogenase is widely studied.

In the cobalt complex of lipoamide dehydrogenase the intensity and contour of the bands at 350 and 440 nm and the fact that the zinc complex generates similar features suggest that they are perturbed flavine transitions. The origin of the 650-nm band is less certain. The absence of the 650-nm band in the zinc complex, depicted in Figure 1, suggests that it may be a cobalt d–d transition, even though the absorptivity is somewhat higher than one commonly encounters for such transitions. On the other hand, tetrahedral copper complexes frequently have absorptivities exceeding 10^3 M^{-1} cm^{-1}. The oxidative properties of Cu(II) precluded its use in this study (Veeger and Massey, 1962). The intensity of the 650-nm band

can also be explained by distorted or entatic configurations of the ligands (Vallee and Williams, 1968). If one attributes the 650-nm band of the cobalt complex to charge transfer transitions, the question why zinc and nickel complexes lack it is not adequately answered. Furthermore, the cobalt complex is not analogous to that of a "radical chelate" because other metals do not generate the band. In inorganic complexes, the resultant geometries usually approximate a tetrahedron or an octahedron. All d–d transitions are parity forbidden in the visible region, but the low tetrahedral field-splitting energy gives rise to long-wavelenth, low intensity ($100 < E^M < 1000$) bands. Among other biologically important transition metals, Fe(II) also has complicated complexation properties and gives colored sytems (particularly heme- and iron-sulfur) whose preferred coordination geometries include square planar and bipyramidal, in addition to the two mentioned above. Manganous ions and Zn(II) are probably closest to cobalt in having only tetrahedral or octahedral preferences in inorganic complexes. Mn(II) d–d transitions, however, are not only parity forbidden but spin forbidden as well (d^5 configuration). Their absorption band intensities would be two orders of magnitude less than those for cobalt making them hard to detect (Cotton and Wilkinson, 1967). Zn(II) is diamagnetic and the only metal in the series where d–d transitions are nonexistent (d^{10} configuration). It appears therefore that the 650-nm band in the cobalt–enzyme complex may well be a d–d transition. Stein and Stein (1971) have obtained a cadmium derivative of the enzyme. Visible absorption spectra show that, whereas the arsenite-modified enzyme shows no change from the native, the cadmium–enzyme exhibits characteristic shifts. These changes, however, are similar to those produced by Cu(II) and phenylmercuric acetate (Casola and Massey, 1966), and we have not observed any induction of optical activity upon addition of Cd to the enzyme.

Return of native structured features in the 450-nm absorption band of the cobalt complex after treatment of the complex with phenanthroline suggests that no drastic alterations of the flavine binding site and flavine configuration have occurred and that the protein or flavine architecture necessary for complex formation approximates the native architecture of the flavine-binding sites. The rationale for this statement is that large increases in band intensity and contour occur in the optical activity of free flavine upon binding to lipoamide dehydrogenase. Those changes are unique to this enzyme. Therefore, any gross alteration of flavine configuration would be expected to produce considerable diminution in band intensity, rather than the shifts which are always observed in the metal complexes discussed here. The irreversible loss of enzyme activity, however, is evidence of a permanent alteration to the active site. Gross conformational changes and the possibility of dimer dissociation have been ruled out by the ultraviolet CD spectrum and ultracentrifugal sedimentation analysis, which demonstrate the gross structural identity of the native, the cobalt complex, and phenanthroline-treated cobalt–enzyme. Apropos of the loss of enzyme activity after Co treatment is the effect of oxygen on lipoate reductase activity. The Co^{II}_2LipDH complex has been assayed immediately upon aeration and after 4 hr of oxygen equilibration. The activity of the complex immediately after aeration is approximately 30% of the original; after 4–7 hr in air, the activity was reduced to 1–5%. This behavior rules out involvement of unmodified native enzyme, since the activity would increase as any native reduced-enzyme oxidizes.

The air-equilibrated transhydrogenase activity on the contrary increases. The immediate and equilibrated transhydrogenase activities vary from 10 to 40% of the original. The DCIP diaphorase decreases over 4 hr to 50% of the original native activity. The oxygen equilibration of the cobalt-modified enzyme is reflected in the spectral character of the 440- and 650-nm bands. For one particular experiment E^M_{440} under anaerobic conditions is 12,400, and after equilibration with O_2 over 5 hr the extinction increases to 13,400. Table I lists the decrease in E^M_{650}. On the average, the decrease in 650-nm band intensity with O_2 equilibration is about 26%, and the band is stabilized in 3 or 4 hr. The enzyme reduced with equimolar NADH gave similar results. Since major spectral changes occur abruptly with the introduction of cobalt and further transient, minor changes occur upon oxidation, it is probable that at least two reactions are initiated concomitant to cobalt complexation. We make this point to lay basis for an understanding of the phenomenon expressed in Figure 2, namely that the pK for cobalt complexation is not coincident with the pK for loss of lipoate reductase activity. This fact is further amplified in the behavior of the cobalt reaction with enzyme reduced by NADH or dihydrolipoate.

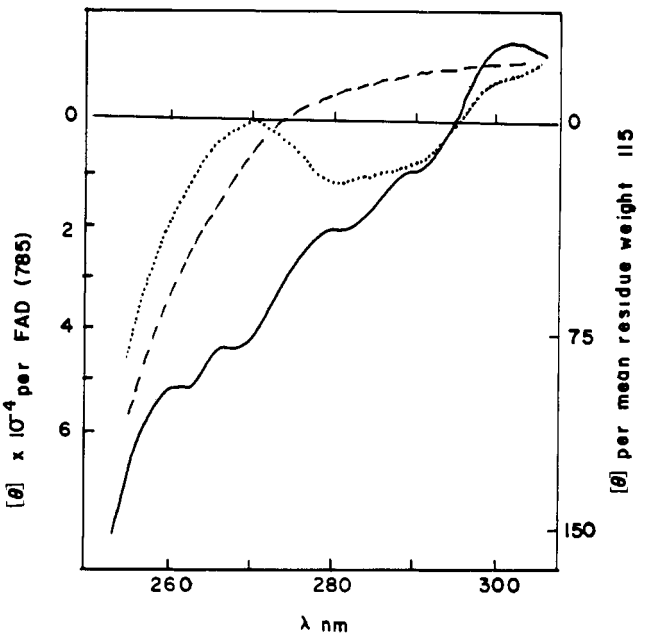


FIGURE 4: Ultraviolet circular dichroism of cobalt–lipoamide dehydrogenase (——), cobalt complex after treatment with phenanthroline (- - - - -), and the native enzyme (· · · ·).

The dihydrolipoate-reduced enzyme does not generate the 650-nm band with Co under conditions where NADH reduction does, and NAD^+ is not the cause of this difference. That NAD^+ is not itself responsible for this difference can be demonstrated in two ways. (1) Recalling that upon NADH reduction of the flavine 1 mol of NAD^+/mol of FAD^+ is bound to the semiquinone and then released upon reoxidation, we can suppose that cobalt complexation somehow requires the presence of NAD^+. However, if cobalt–lipoamide dehydrogenase at pH 7 is dialyzed to remove NAD^+ and allowed to react with NaCN to attempt formation of NAD^+–CN^-, no band is generated at 325 nm (Colowick *et al.*, 1951a,b). (2) More conclusive evidence required treatment of cobalt–lipoamide dehydrogenase with *Neurospora crassa* nicotinamide adenine dinucleotidase. No effect was observed on the intensity of the 650-nm band. Massey and Williams (1965) have indicated

TABLE III: Modified Activities of Lipoamide Dehydrogenase.

	Arsenite-Treated	Cobalt-Treated
Lipoate reductase	Absent[a]	Absent
Transhydrogenase	Decrease[a]	Decrease
Diaphroase (DCIP)	30-Fold increase[b]	Decrease by 50%

[a] Massey and Veeger (1961). [b] Veeger and Massey (1962).

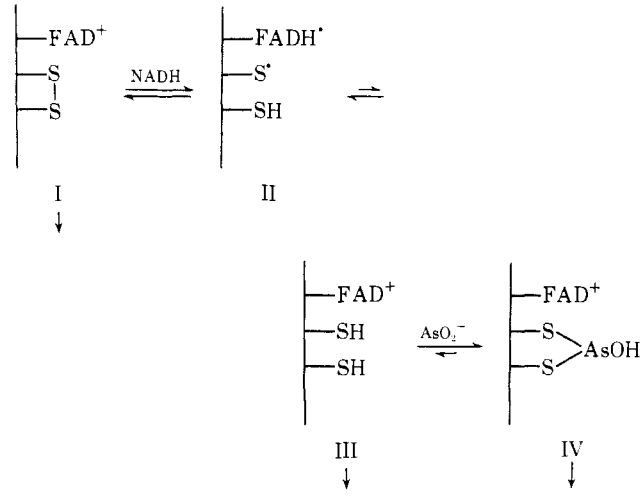
that enzyme-bound NAD^+ is not a good substrate for NADase, but 50 min was sufficient time for NADase to hydrolyze the enzyme-bound NAD^+. Although a 2-hr reaction time was allowed, it is conceivable that Co-complexed NAD^+ may be an even poorer substrate.

Nevertheless, considering that NAD^+ may not be involved in the dialyzed complex, it seems reasonable to accept at this point that NAD^+ is not the cause of the difference in cobalt reactivity with the NADH and dihydrolipoate-reduced enzymes; it appears that the result indicates a property intrinsic to the two systems. Brady and Beychok (1971) have shown from purely spectral evidence that the NADH-reduced enzyme differs in the reduced state from the dihydrolipoate-reduced enzyme. We believe that the present data represent the first evidence from chemical reaction of a distinction in these two reduced states.

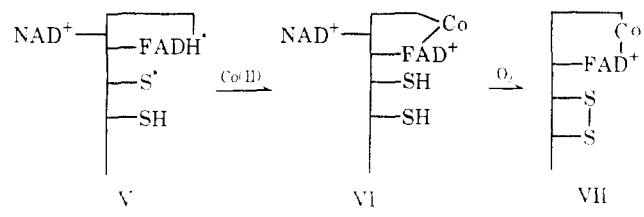
Moreover, the absence of the 650-nm band in the cobalt-treated, dihydrolipoate-reduced enzyme does not mean that cobalt has no effect on that form of the enzyme. For there is no doubt that cobalt protects it from denaturation. When native enzyme is reduced with dihydrolipoate at pH 10 and 4° in the absence of cobalt, it slowly turns yellow giving free FAD^+ and loss of lipoate reductase activity. Subsequent dialysis against pH 7 phosphate buffer yielded a white protein precipitate. On the other hand if cobalt is added to the enzyme immediately after reduction, the protein remains in solution upon dialysis to pH 7. The protein does not turn yellow but persists as a red complex, and retains the equimolar concentration (relative to enzyme flavine) of cobaltous ions added. We are currently studying the properties of this material which may be $Co(II)_2$–apoenzyme. At room temperature the $Co(II)_2$–apoenzyme precipitates. This thermolability is similar to apoenzyme prepared by other methods (Brady and Beychok, 1969).

The most striking feature of the CD spectra of these metal–enzyme complexes is the induction of the strong ellipticity in the vicinity of 450 nm. Most other flavoproteins possess optical activity in this region; for instance mammalian D-amino acid oxidase has a CD spectrum very close to that for Zn_2–LipDH (Aki *et al.*, 1966). Edmondson and Tollin (1971) in their CD analyses of a number of flavoproteins showed that the following ones have optical activity in both the 350- and 450-nm regions: snake venom L-amino acid oxidase, glucose oxidase, spinach ferredoxin–TPNH reductase, clostridial flavodoxin, and the Shethna flavoprotein. These authors suggested the possible classification of oxidases and dehydrogenases according to their optical activity, just as Massey *et al.* (1969) have done from sulfite reactivity data, oxygen reoxidizability, and the nature of the semiquinoid state. Lipoamide dehydrogenase is odd by all means of classification. It possesses no optical activity in the 450 region; the NADH-reduced form is air reoxidizable; the semiquinone state is red but neutral and diamagnetic, and does not react with sulfite. However, the CD spectra of the $Zn(II)_2$- and $Co(II)_2$–LipDH confirm the existence of potential optical activity in the 450-nm region, and this may indicate the close relationship of the active-site configuration to that of the enzymes above.

The usefulness of the cobalt complex of lipoamide dehydrogenase lies in its ability to perturb the FAD^+ binding site of the enzyme in a way that can be monitored by its optical activity. Other modifiers of this enzyme like arsenite (Massey and Veeger, 1961) and cadmium (Stein and Stein, 1971) produce changes in the ultraviolet absorption of the flavine. Little or no effect is seen in the circular dichroism of the modified enzymes. Further, it appears that all modifications up to now are related directly to the reactive disulfide moiety at the active site, inasmuch as these reagents are particularly reactive with sulfhydryl groups. It has not been possible to probe other features of the active site. Using the arsenite enzyme as a model, we can compare the reaction scheme below of Massey and Veeger (1961) to our own data. One



might argue that the cobalt binding site is the same as the arsenite site in the following way. At pH 10, the sulfhydryl groups of form III would be ionized and should be prime candidates for reaction with Co(II). In that case one ought to expect similar behavior in the lipoate reductase, transhydrogenase, and diaphorase activities of the arsenite- and cobalt-treated enzymes. One observes in fact the behavior seen in Table III. The marked differences in DCIP-diaphorase activities indicate that the site of diaphorase activity is not equally affected in the Co and arsenite complexes. The optical activity of the Co(II)–enzyme is further support of this distinction insofar as the altered circular dichroism of the Co(II)–enzyme (Figure 1) is quite marked and that of the arsenite enzyme is hardly affected over that of the native oxidized enzyme. These striking differences would suggest a model of complexation like that below, rather than liganding through SH groups.



Co(II) forms the complex only with the NADH-reduced enzyme; thus the reactive species is V; the role of the bound NAD^+ stabilizing the red semiquinone may be important, or the conformation around the flavine in this reduced species is sufficient to prevent identical complex formation with the dihydrolipoate-reduced enzyme. In VI, after cobalt–complex formation, the flavine is depicted in the oxidized form, considering the similarity of the absorption spectra to that of oxidized flavine. We have not determined the state of the two functional sulfurs. They are depicted in VI as sulfhydryl. But whether they are stable as such, or exist with electron transfer to neighboring amino acid residues, or NAD^+, remains to be determined. Upon aeration, however, they most likely return to the disulfide configuration (VII). Whether the NAD^+ leaves at stage VI or at form VII after O_2 equilibration (where minor spectral alterations occur) is again conjectural. Preliminary efforts at identifying a possible amino acid residue as electron acceptor upon complex formation have not been successful.

It has been suggested that the disulfide is not involved in the DCIP-diaphorase reaction (Searls *et al.*, 1961), the oxidation–reduction being only between the dye and the enzyme–flavine. This would qualitatively agree with an equal or enhanced diaphorase activity found in the arsenite or cadmium complexes, whereas liganding to the flavine in the cobalt complex would interfere with the diaphorase activity.

The p*K* of 8.25 for cobalt complex formation is within range of a mercaptide, particularly the free sulfhydryl of the semiquinone–mercaptide diradical, but chemical modification studies, in progress, are needed to determine requirements for complexation. The inflexion curve in Figure 2 for complex formation cannot at this time be used to suggest that only one ionizing group is responsible for this pH behavior, because it is not an equilibrium curve. Each point on the curve represents a new sample of enzyme reduced with NADH at the designated pH, treated with cobalt, and then reoxidized and returned to pH 7 to rule out any pH dependence of the absorption maximum. While each sample has been treated similarly, the reoxidation must be regarded as stopping any reaction between cobalt and enzyme. We have not drawn, therefore, a theoretical titration curve through the experimental points. Strictly speaking, successive points on the curve do not represent different equilibria in the cobalt–enzyme association, and we cannot properly call the inflexion point of the curve a p*K*. However, since preparations have been similarly treated, presumably only changes in the cobalt–enzyme equilibria are reflected in changing extinction at 650 nm.

In summary, a cobalt–lipoamide dehydrogenase complex has proved significant in demonstrating chemically structural differences between the NADH- and dihydrolipoate-reduced enzyme, has elicited optical activity from a previously quiescent 450-nm absorption band, and has shown that a flavoprotein with no metal requirement can form a specific metal complex, other than by inserting between the catalytic disulfide, in such a way that normal enzyme activity is blocked. The last of these points offers the possibility of using this complex to explore the path of electron transfer in flavoenzymes and to establish common structural properties in the vicinity of bound flavine.

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