Cobalt(II)-Substituted Class III Alcohol and Sorbitol Dehydrogenases from Human Liver†

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ABSTRACT: The catalytic zinc atoms in class III (χ) alcohol dehydrogenase (ADH) and sorbitol dehydrogenase (SDH) from human liver have been specifically removed and replaced by cobalt(II) with a new ultrafiltration technique. The electronic absorption spectrum of class III cobalt ADH (ϵ_{638} = 870 M^{-1} cm^{-1}) is nearly identical with those of active site substituted horse EE and human class I ($\beta_1\beta_1$) cobalt ADH. Thus, the coordination environment of the catalytic metal is strictly conserved in these enzymes. However, significant differences are noted when the spectra of class III ADH·coenzyme complexes are compared to the corresponding spectra of the horse enzyme. The spectrum of class III ADH·NADH is split into three bands, centered at 680, 638, and 562 nm. The class III ADH·NAD^+ species resembles the alkaline form of the corresponding horse enzyme complex but without exhibiting the pH dependence of the latter. These spectral changes underscore the role of the coenzymes in differentially fine tuning the catalytic metal for its particular function in each ADH. The noncatalytic zinc of class III ADH exchanges with cobalt at pH 7.0. While 9 residues out of 15 in the loop surrounding the noncatalytic zinc of class III ADH differ from those of the class I ADH, the electronic absorption spectra of cobalt in the noncatalytic metal site of class III ADH establish that the coordination environment of this site is conserved as well. The spectrum of cobalt SDH differs significantly from those of cobalt ADHs. Comparison of absorption maxima corroborates the recent proposal [Eklund, H., Horjales, E., Jörnvall, H., Brändén, C.-I., & Jeffery, J. (1985) *Biochemistry 24*, 8005–8012] that in SDH only one cysteine is a metal ligand. The relatively low molar absorptivity of cobalt SDH (ϵ_{562} = 230 M^{-1} cm^{-1}) indicates a distorted tetrahedral or pentacoordinate structure of the metal ion. Binding of NADH to cobalt SDH induces a transition to a tetrahedral metal site as manifested by a hyperchromic effect on the main absorption band (ϵ_{590} = 410 M^{-1} cm^{-1}). This perturbation is unique when compared to cobalt ADHs and represents yet another type of coenzyme-induced modulation of the catalytic metal in this family of enzymes.

Human alcohol dehydrogenases (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1; ADH)[1] constitute a family of dimeric enzymes encoded by at least five genes. The primary structures of class I (α, β, γ), class II (π), and class III (χ) ADHs have been determined (Jörnvall et al., 1987a; Höög et al., 1987; Kaiser et al., 1988). Since sequence homology among the three classes is only 60%, they have now been considered different enzymes rather than isozymes (Jörnvall et al., 1987b). Each one of the five types of subunits binds two zinc ions. The protein ligands of the catalytic zinc, i.e., one histidine and two cysteines, and of the noncatalytic zinc, i.e., four cysteines, are conserved in all three classes despite numerous amino acid replacements in the domains surrounding these regions.

The tetrameric sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14; SDH) is structurally related to the ADHs (Jeffery et al., 1981). SDH has recently been isolated from human liver (Maret & Auld, 1988) and sequenced (Karlsson et al., 1989). Residue identity with class I ADHs is approximately 25%. Two important observations were made in relationship to the metal-binding properties of SDH. Three of the four cysteine ligands of the noncatalytic zinc of the ADHs are absent in SDH, leading to a stoichiometry of only one zinc per subunit. Furthermore, a comparison of the primary sequence of sheep liver SDH with the three-dimensional structure of horse liver ADH has led to the suggestion that in SDH a glutamate ligand replaces one of the two cysteine ligands of the catalytic zinc in ADHs (Eklund et al., 1985).

The horse EE enzyme, representing class I ADHs, is the only tertiary structure which has been determined by X-ray crystallography (Brändén et al., 1975). It has, therefore, been widely used as the frame of reference for discussions of the structure–function relationships of ADHs. The limitations of this model, however, become obvious when the diversity of structures described above is considered. Consequently, alternative approaches are needed to study the metal-binding sites of these enzymes. Replacement of intrinsic zinc by cobalt(II) has been used extensively to characterize the zinc coordination in such enzymes and to report on structural changes of the metal upon interaction with substrate and coenzyme. Here, metal substitution is used to characterize and compare the active sites of class III ADH and SDH from human liver for which structural information from X-ray diffraction analyses is not yet available.

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[1] Abbreviations: ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Btp, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DEAE, diethylaminoethyl.

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Experimental Procedures

Materials. Dipicolinic acid was obtained from Sigma Chemical Co. (St. Louis, MO); cobalt sulfate and cobalt chloride, both Specpure grade, were from Johnson Matthey Chemicals Ltd. (London, England); NADH, grade I, and LiNAD$^+$ were from Boehringer Mannheim Biochemicals (Indianapolis, IN); sodium hydroxide, Suprapur grade, was from Merck (Darmstadt, FRG). Nitrogen gas from a liquid nitrogen tank was further purified by high-capacity and indicating oxygen traps from Pierce Chemical Co. (Rockford, IL). All experiments with the cobalt-substituted enzymes were performed with nitrogen-saturated buffers and by blanketing solutions with nitrogen. For dialysis experiments a DiaCell system from Instrumed Inc. (Union Bridge, MD) was used.

Preparation of Sorbitol Dehydrogenase. The purification and the assay of human liver sorbitol dehydrogenase were performed as published (Maret & Auld, 1988). Since multiple forms of SDH are resolved during the last chromatographic step (Maret & Auld, 1988), only chromatographically pure fractions were taken for the metal-exchange experiments.

Preparation of Class III Alcohol Dehydrogenase. The enzyme was purified and assayed as described (Wagner et al., 1984) and stored in liquid nitrogen. A final purification was achieved by HPLC (Jean-Marc Moulis, personal communication) with a Waters Protein Pak DEAE 5PW semipreparative column (21.5 × 150 mm) operated at a flow rate of 3 mL/min. The buffer system used was 10 mM Tris-HCl, pH 7.8–0.05 mM dithiothreitol (buffer A) and the same buffer containing 100 mM sodium chloride (buffer B). The gradient program with linear segments was 10 min at 100% A, 20 min at 80% A, 70 min at 40% A, and 100 min at 0% A with the times given as end times. This system provides sufficient separation of the different forms of class III ADH (Dafeldecker & Vallee, 1982; Beisswenger et al., 1985; Valkonen & Goldman, 1988). Only the first-eluting peak representing the major form (χ_1) was pooled and used for the present study. The specific activity was ≥0.6 unit/mg (Wagner et al., 1984).

Cobalt(II)-Substituted Class III Alcohol Dehydrogenase. All metal-exchange experiments were performed at 4 °C by ultrafiltration using Centricon 30 microconcentrators from Amicon Corp. (Danvers, MA) under operating conditions specified by Amicon.[2]

Substitution of catalytic zinc was achieved by preparation of the apoenzyme and subsequent insertion of cobalt. About 6 mg of the enzyme in 2 mL of 33 mM Tes, pH 7.4 (4 °C), was concentrated to about 50 μL, taken up in 2 mL of the same buffer containing 10 mM dipicolinic acid, incubated for 1 h, and concentrated, and the procedure was repeated twice, except that the third incubation was for 16 h. After the removal of a small amount of white precipitate by centrifugation, the sample was transferred to a new microconcentrator and subjected to a fourth wash with an incubation of 10 min. Subsequently, the enzyme was washed four times with the same buffer without dipicolinic acid to remove the chelating agent. Cobalt(II) chloride in 33 mM Tes, pH 7.7 (2 mL of 1 mM), was then added and the sample incubated for 30 min. Excess cobalt was removed by two washes with Tes buffer. To study the influence of pH on the electronic absorption spectra, the pH was adjusted by consecutive washes in the microconcentrators with the following buffers: 0.1 M Mes (pH 5.9 and 6.3); 33 mM Tes (pH 7 and 7.7); 0.1 M Tris (pH 8.3 and 8.7); 0.1 M Btp (pH 9.6).

[2] Pretreatment of the Centricon microconcentrators as recommended by Auld (1988) or by multiple washes with buffers containing 10 mM dipicolinic acid is necessary to avoid zinc contamination of the samples.

The noncatalytic zinc was substituted by direct exchange. About 5.5 mg of the enzyme was brought into 0.1 M Tes, pH 7.0, and concentrated. The enzyme was washed two times with 2 mL of 0.2 M cobalt chloride in the Tes buffer and subsequently six times with the cobalt-free Tes buffer. Accordingly, the enzyme was only exposed to the high concentrations of cobalt during centrifugation for about 1 h. Subjecting the enzyme to this treatment results in a degree of substitution of 43%. The enzymatic activity was assessed in 0.1 M Tris-HCl, pH 9.0 (0.5 M ethanol; 1.5 mM NAD$^+$), and compared to the activity of the starting material.

Cobalt(II)-Substituted Sorbitol Dehydrogenase. SDH, 6 mg, in 0.1 M Hepes, pH 7.0, was concentrated and treated as follows: (i) five washes with 2 mL of 10 mM sodium phosphate–10 mM dipicolinic acid, pH 7.0, (ii) five washes with 2 mL of 0.1 M Hepes, pH 7.0, to remove the excess of chelating agent, (iii) 10-min incubation with a 5-fold molar excess of cobalt(II) sulfate in 2 mL of the same buffer, (iv) concentration, and (v) two washes with 2 mL of the buffer to remove excess cobalt. The residual enzymatic activity of the apoenzyme was determined in glycine buffer pretreated with dithizone before pH adjustment with sodium hydroxide (Holmquist, 1988).

Electronic Absorption Spectroscopy. Absorption spectra were recorded at 10 °C with a Cary 219 spectrophotometer controlled by an Apple IIe computer with software provided by Varian Associates, Inc. (Palo Alto, CA). Spectra were measured on samples of 100 μL in masked microcells, filled and stoppered under a nitrogen atmosphere. Molar absorptivities are based on the cobalt content of the enzymes.

Metal Analyses. The zinc and cobalt contents of enzyme and buffer samples were determined by flameless atomic absorption spectrophotometry with a Perkin-Elmer Model 5000 spectrophotometer equipped with an HGA 500 graphite furnace programmer and an AS 40 autosampler. Metal-to-protein stoichiometries are based on the protein concentration determined by a Coomassie Blue protein–dye binding assay (Scopes, 1982) and a subunit molecular weight of 37 700 for SDH (Maret & Auld, 1988) and of 40 000 for class III ADH (Wagner et al., 1984).

Results

Metal Exchange in Centrifugal Microconcentrators. A procedure was developed that allows for metal-exchange experiments with relatively small amounts of proteins (2–6 mg). The present technique utilizes centrifugal microconcentrators, can be performed in 1–2 days, and circumvents lengthy dialysis steps. The microconcentrators are easily manipulated under a nitrogen atmosphere. In the ultrafiltrate, the removal of zinc form the enzymes has been followed by zinc analyses and the removal of the excess dipicolinic acid (ϵ_{271} = 4500 M^{-1} cm^{-1}) by spectrophotometry. Such determinations showed that with SDH (Figure 1) three washes are sufficient to remove both the catalytic zinc and dipicolinic acid, respectively. The ultrafiltration technique was also employed for preparation of both class III cobalt-hybrid ADHs. One hybrid enzyme was obtained by extracting the catalytic zinc ions with dipicolinic acid in solution and subsequent reconstitution of the apoenzyme with cobalt(II) under conditions similar to those described for the preparation of cobalt SDH. The other class III cobalt-hybrid ADH was prepared by direct metal exchange. In this procedure zinc in the noncatalytic site is displaced by a large excess of cobalt(II) without forming an intermediate apoenzyme (Sytkowski & Vallee, 1976). The metal analyses of the different species are given in Table I. Metal-to-protein stoichiometries that are slightly above the theoretical value

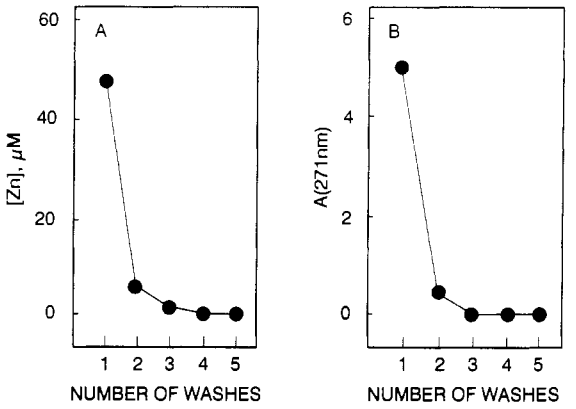

FIGURE 1: Removal of zinc (A) monitored by atomic absorption spectrophotometry and of excess of dipicolinic acid (B) followed by electronic absorption spectroscopy from human liver sorbitol dehydrogenase. The enzyme, 4 mg, was subjected to five consecutive washes with buffer containing dipicolinic acid (A) and buffer without dipicolinic acid (B) in centrifugal microconcentrators as described under Experimental Procedures.

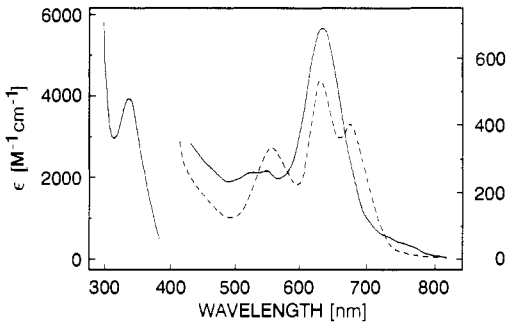

FIGURE 2: Electronic absorption spectra of cobalt at the active site of class III alcohol dehydrogenase (solid line) and of the complex of the enzyme with NADH (dashed line) in 33 mM Tes, pH 7.7. The concentrations of enzyme and NADH were 122 μM and 3.2 mM, respectively. The metal analyses of this enzyme yielded 0.9 mol of cobalt and 1.9 mol of zinc.

Table I: Metal Content of Class III Cobalt-Hybrid Alcohol Dehydrogenases, Cobalt Sorbitol Dehydrogenase, and the Corresponding Apoenzymes

species	metal content per subunit		
	Co	Zn	total
class III apo-ADH		1.0	1.0
class III cobalt ADH			
substituted at catalytic site	0.6	1.6	2.2
	0.9	1.9	2.8
	0.4	1.9	2.3
substituted at noncatalytic site	0.4	1.9	2.3
cobalt SDH	0.9	0.1	1.0
	0.7	0.4	1.1
apo-SDH		0.1	0.1

of 2:1 were determined for the class III cobalt-hybrid ADHs. An elevated zinc content was also reported for class I ($\beta_1\beta_1$) cobalt-hybrid ADH (Schneider-Bernlöhr et al., 1988). One zinc ion per subunit and a specific enzymatic activity of 13% of that of the starting material were measured for the class III apoenzyme. For SDH the residual activity of the apoenzyme was 8%, in close agreement with a zinc analysis of 0.1 mol. Upon addition of cobalt(II) to apo-SDH the enzymatic activity increased to 81%. An increase in activity was also noted when the class III apoenzyme was reconstituted with cobalt(II). However, the present analytical data do not allow one to determine whether the enzymatic activity of the class III cobalt-hybrid ADH is slightly higher or lower than that

Table II: Absorption Maxima and Molar Absorptivities for Cobalt-Substituted Class III Alcohol Dehydrogenases and the Complexes with Coenzymes

species	absorption max (nm)	ε (M^{-1} cm^{-1})
catalytic site[a]		
enzyme	638	870
	552	480
	528	440
	340	4830
enzyme + NADH	680	440
	638	590
	562	450
enzyme + NAD^+	680	300
	635	600
	562	290
noncatalytic site		
enzyme	740	230
	638	460
	340	5620

[a] The molar absorptivities are based on a stoichiometry of one cobalt per subunit calculated from the metal content per active site and are averages of three preparations for the enzyme and of two preparations of the binary complexes.

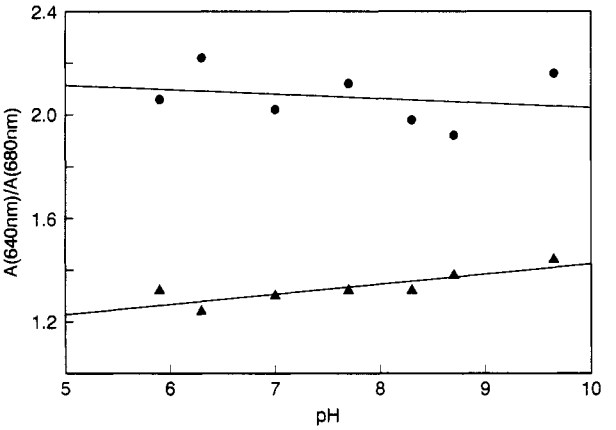

FIGURE 3: pH dependence of the electronic absorption spectra of active site substituted class III cobalt alcohol dehydrogenase with NADH (triangles) and NAD^+ (circles). A spectral change as a function of pH is expected to affect absorption maxima. Therefore, ratios between absorption maxima were determined and plotted in this experiment.

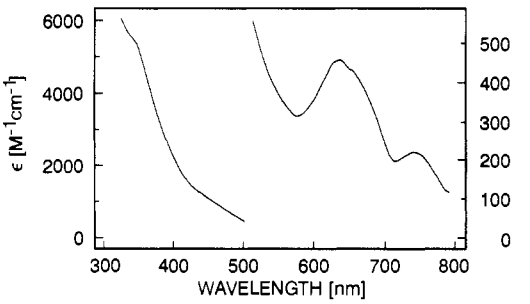

FIGURE 4: Electronic absorption spectrum of cobalt at the noncatalytic site of class III alcohol dehydrogenase in 0.1 M Tes, pH 7.0. The degree of substitution was 43%. The spectrum was measured at an enzyme concentration of 0.4 mM.

of the zinc enzyme. The class III cobalt-hybrid ADH prepared by direct metal exchange had unchanged enzymatic activity.

Cobalt Class III Alcohol Dehydrogenases. The electronic absorption spectrum of the active site substituted cobalt enzyme (Figure 2, solid line) is characterized by a major band at 638 nm and two minor ones at 528 and 552 nm. The binding of NADH (Figure 2, dashed line) splits the main visible absorption band into three components at 562, 638, and 680 nm. A ligand-to-metal charge-transfer band at 340 nm present in the enzyme is obscured by the presence of NADH

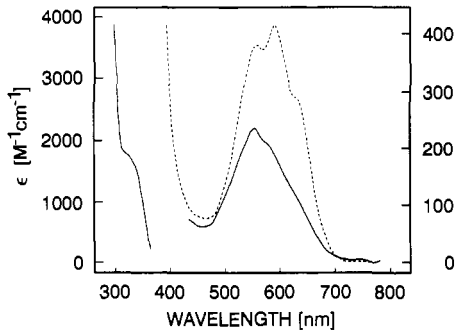

FIGURE 5: Electronic absorption spectra of cobalt(II)-substituted sorbitol dehydrogenase (solid line) and its complex with NADH (dashed line) in 0.1 M Hepes, pH 7.0. The metal analyses of this enzyme yielded 0.7 mol of cobalt and 0.4 mol of zinc. The concentrations of enzyme and NADH were 0.3 and 9 mM, respectively.

in the binary complex. The addition of NAD^+ (150-fold molar excess) to the enzyme produces a similar perturbation with only marginal differences with regard to the absorption maximum, i.e., 635 nm as compared with 638 nm in the NADH complex, and the intensities of the satellite bands (see Table II). The coenzyme-perturbed spectra are pH-independent between pH 5.9 and pH 9.6 (Figure 3).

The electronic absorption spectrum of cobalt in the noncatalytic site (Figure 4) differs from that of cobalt in the catalytic site (Figure 2, solid line). It has a characteristic absorption band at 740 nm in addition to the band at 638 nm, bearing a shoulder toward longer wavelengths, and the charge-transfer band at 340 nm. Absorption maxima and molar absorptivities of both species are listed in Table II.

Cobalt Sorbitol Dehydrogenase. Cobalt SDH exhibits an electronic absorption spectrum (Figure 5, solid line) with maxima at 562 nm (ϵ = 230 M^{-1} cm^{-1}) and at 320 nm (ϵ = 1750 M^{-1} cm^{-1}). In the presence of NADH, the intensity of the main visible absorption band is increased almost 2-fold (ϵ_{590} = 410 M^{-1} cm^{-1}), and the band is split into three components at 560, 590, and 625 nm (Figure 5, dashed line). These components are seen as shoulders in the enzyme when NADH is absent.

Discussion

Technique of Metal Exchange. Site-specific metal exchange in alcohol dehydrogenases is designed to yield hybrid enzymes substituted at either the catalytic or noncatalytic site (Sytkowski & Vallee, 1976; Maret et al., 1979). The catalytic zinc in human liver class III ADH can be removed specifically with dipicolinic acid in solution to yield an apoenzyme with low residual enzymatic activity and with the noncatalytic zinc still present. Reconstitution of this enzyme is then readily achieved simply by adding cobalt(II). The conditions for the replacement are similar to those reported for class I $\beta_1\beta_1$ ADH (Schneider-Bernlöhr et al., 1988). The single zinc in SDH was substituted also by an analogous procedure. It was noticed that class III apo-ADH has a relatively high residual enzymatic activity of 13%. A similar observation has been made for the human $\beta_1\beta_1$ isozyme treated with dipicolinic acid in solution (Schneider-Bernlöhr et al., 1988), and it was suggested that the noncatalytic sites are replenishing the zinc-depleted catalytic sites. The low enzymatic activity of the corresponding horse liver apoenzyme prepared in crystal suspensions (Maret et al., 1979) may reflect differences in metal exchange between crystals and solution, stabilities of the apoenzymes, or affinities of the sites for zinc. To effect specific exchange of the noncatalytic zinc of class III ADH, the enzyme merely had to be incubated with excess cobalt(II) at neutral pH. It is then not necessary to lower the pH to assist metal exchange at this site as was initially required for horse liver ADH (Sytkowski & Vallee, 1976).

Class III Alcohol Dehydrogenase. (A) Catalytic Metal Site. The effect of coenzyme on the electronic absorption spectra of cobalt(II)-substituted class III ADH and the absence of absorbance around 740 nm, typical when cobalt is at the noncatalytic site (Figure 4), establish that the procedure using dipicolinic acid only replaces the catalytic zinc. There are a number of striking results: (i) In the absence of coenzyme the spectrum of the enzyme is virtually identical with that of active site substituted horse liver EE (Maret et al., 1979) or human liver $\beta_1\beta_1$ (Schneider-Bernlöhr et al., 1988) cobalt ADH, with the noticeable difference of a 10–12-nm blue shift of the main absorption maximum in class III cobalt ADH. In $\beta_1\beta_1$ cobalt ADH absorption bands around 520 nm were not reported (Schneider-Bernlöhr et al., 1988). Such a conservation of coordination environment is surprising in view of the many amino acid substitutions near the active site in class III ADH. Therefore, not only are one histidine and two cysteine ligands to the metal conserved (Kaiser et al., 1988), but also a very similar structure is retained. (ii) The perturbation of the spectrum by coenzymes differs from that of other ADHs and SDH (see below). The spectrum of class III cobalt ADH with NADH is very similar to that with NAD^+, and both resemble that of the alkaline form of the complex of the horse enzyme with NAD^+ (Maret & Zeppezauer, 1986). While the latter shows a pH-dependent transition with a pK_a of 6.9, the spectra of the class III enzyme complexes with either coenzyme form are independent of pH between 5.9 and 9.6. NADH binding to horse liver cobalt ADH shifts the main absorption band from 650 to 680 nm (Maret, 1980). The spectrum is not split as that of the class III enzyme. These results clearly show that the coenzyme differentially alters the structure of the catalytic metal atom in these ADHs as indicated also by the absence of a detectable ionization controlling the spectrum of the class III enzyme·NAD^+ complex. An ionization ($pK_a \sim 7.6$) in the horse liver zinc ADH·NAD^+ complex has repeatedly been attributed to the metal-bound water molecule [for a review, see Pettersson (1987)], and most mechanisms proposed for this enzyme have evolved from this assignment. A similar ionization ($pK_a \sim 6.9$) was shown to control binding of NAD^+ to horse liver cobalt ADH (Maret & Zeppezauer, 1986). Making the assumptions that (i) a water molecule is bound to the metal in the class III enzyme and (ii) an ionization of this water molecule will be detected in the spectra of the cobalt enzyme, the pH independence of the spectra of the class III enzyme with bound NAD^+ casts doubt on the assignment of the pK_a of 7.6 to the ionization of the metal-bound water molecule in horse liver ADH.

If the close similarity between class III cobalt ADH·NAD^+ and the alkaline form of the corresponding complex of horse liver ADH indeed reflects a common structural feature, the very weak inhibition of the class III enzyme by pyrazole (Wagner et al., 1984) can be explained readily: The binding of pyrazole requires the protonated form of the enzyme (Andersson et al., 1981), which is unavailable in the class III enzyme due to its peculiar pH dependence.

(B) Noncatalytic Metal Site. In Figure 6 the protein sequences around the noncatalytic zinc atom are compared for the three classes of human ADH, the horse EE enzyme, and human SDH. Despite numerous amino acid changes in the class III enzyme, the close correspondence of the electronic spectrum of cobalt at this site to that of horse liver cobalt ADH with substitution at the noncatalytic metal site (Sytkowski &

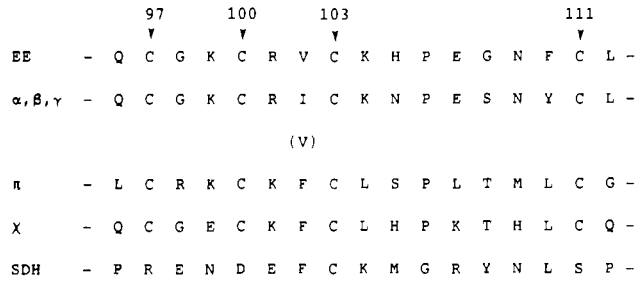

FIGURE 6: Protein sequences of human and horse (EE) alcohol dehydrogenases in the loop holding the noncatalytic metal. The cysteine ligands are indicated by arrowheads [from Jörnvall et al. (1987c) and Kaiser et al. (1988)]. The sequence from the corresponding region in the structure of human liver sorbitol dehydrogenase is also given for comparison, demonstrating the lack of three of the four cysteine ligands of alcohol dehydrogenases (Karlsson et al., 1989). The only difference in the three class I alcohol dehydrogenase sequences in this region is an Ile to Val substitution at position 102 in the β-subunits.

Vallee, 1976) establishes that the geometry of this metal site is also highly conserved. However, it appears that the amino acid replacements affect the metal exchange since the noncatalytic zinc of class III ADH exchanges at neutral pH, whereas previous metal exchange at this site was only successful at pH values below 6 (Sytkowski & Vallee, 1976).

Sorbitol Dehydrogenase. The maximum of the electronic absorption spectrum of cobalt SDH is at a higher energy than that of active site substituted cobalt ADH (Maret et al., 1979) including class III ADH. Two empirical rules have been applied to characterize the coordination environment and to estimate the number of cysteine ligands in cobalt-substituted metalloenzymes: (i) The molar absorptivity of the near-ultraviolet sulfur–metal charge-transfer bands has been correlated with the number of cysteine ligands. The range of values, 900–1300 M^{-1} cm^{-1} per cobalt–thiolate bond (May & Kuo, 1978), is only approximate since the geometry of the coordination strongly influences the intensity of the bands. The absorbance at 320 nm in cobalt SDH is typical for sulfur–metal charge transfer in cobalt–thiolate complexes, and a distinction between one or two thiolate ligands cannot be made on the basis of a molar absorptivity of 1750 M^{-1} cm^{-1} (Figure 5). (ii) The spectrochemical series predicts that the main visible absorption band will red shift upon increasing the number of cysteine ligands. The blue shift of the main absorption band of cobalt SDH in comparison with those of other cobalt ADHs with two or four cysteine ligands then supports the idea that only one cysteine is bound to the catalytic metal in SDH. In this regard the overall spectrum of cobalt SDH is very similar to that of the ES^1 form of cobalt-substituted β-lactamase II from *Bacillus cereus* (Bicknell et al., 1986), an enzyme in which there is only one cysteine ligand to the metal (Davies & Abraham, 1974; Sutton et al., 1987).

These experiments provide direct evidence for an altered zinc ligand sphere in human liver SDH much as this was hypothesized earlier. A structural model for sheep liver SDH based on the three-dimensional structure of horse liver ADH (Eklund et al., 1985) predicts that in SDH the catalytic zinc ligand cysteine-174 of ADH is changed to a glutamic acid residue in spite of the fact that a cysteine at a corresponding position could be present when the sequence of SDH is aligned differently. The latter situation was unsatisfactory in that it created a large gap in a presumably conserved helix. Hence, the change of a metal ligand was deduced by obtaining the best fit of the primary structure of SDH to the three-dimensional model of horse liver ADH. The replacement of a cysteine ligand supported by the present data is surprising in light of the strict conservation of the metal coordination in all three classes of liver and of yeast ADH. However, alignments of the primary structures of *Thermoanaerobium brockii* ADH (Peretz & Burstein, 1989) and *Escherichia coli* L-threonine dehydrogenase (Aronson et al., 1989) with those of SDH and liver ADH indicate an aspartic acid residue at position 174. Therefore, a variation at this position may be a more general occurrence. Though absorption spectroscopy cannot identify a glutamate ligand to cobalt, a coordination of the catalytic metal in SDH to a histidine, a cysteine, and a glutamate is consistent with all available data.

The spectral changes observed upon binding of NADH to cobalt SDH (Figure 5) are thought to reflect a transition to a tetrahedral metal coordination. This is borne out by the appearance of a split in the main absorption band as well as an increase in its intensity [for a review, see Banci et al. (1982)]. Such hyperchromicity is not observed in any of the cobalt ADHs and points to a unique structural transition.

Effect of Coenzymes on Metallodehydrogenases. Binding of NADH to horse liver ADH elicits a large conformational change described as a domain movement (Eklund & Brändén, 1987). However, it is not yet known which amino acids are crucial for the coupling between the coenzyme-binding domain and the structural change of the metal complex in the catalytic domain. Two hinges, comprising residues 173–175 and 318–321, connect these domains (Colonna-Cesari et al., 1986). The protein sequences GCG of the first hinge and GG of the second hinge are conserved in all three ADH classes but not in SDH (Jörnvall et al., 1987c; Kaiser et al., 1988). The first hinge includes cysteine-174, the ligand of the catalytic metal in ADH that is replaced in SDH. In addition, in class III (and II) ADH tryptophan substitutes for phenylalanine found in class I ADHs at position 321 in the second hinge. The present investigation establishes that coenzyme binding also leads to a structural change at the catalytic metal of class III ADH and SDH. It is assumed that the substitutions in the hinges alter the induction of the conformational change and are, therefore, related to the differences in geometries of the catalytic metal complex observed in the enzymes with bound coenzymes.[3] Since only 22 residues out of 374—half of which are glycine—are conserved in this family of enzymes (Jörnvall et al., 1987c), the occurrence of a structural change of the catalytic metal in class III ADH and SDH is striking and most certainly indicates a coenzyme-induced conformational realignment of the enzyme as a common mechanistic requirement.

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Registry No. NADH, 58-68-4; NAD, 53-84-9; cysteine, 52-90-4.

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[3] Another critical region of the coenzyme binding domain comprising residues 294–298 differs in class II and class III ADH and SDH. This region has been designated a "wedge" which is thought to move upon coenzyme binding so that it can accommodate a region of the catalytic domain (Eklund & Brändén, 1987).

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Thermodynamics of Protein–RNA Recognition in a Highly Conserved Region of the Large-Subunit Ribosomal RNA†

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ABSTRACT: Ribosomal protein L11 from *Escherichia coli* specifically binds to a highly conserved region of 23S ribosomal RNA. The thermodynamics of forming a complex between this protein and several different rRNA fragments have been investigated, by use of a nitrocellulose filter binding assay. A 57-nucleotide region of the RNA (C1052–U1108) contains all the protein recognition features, and an RNA fragment containing this region binds L11 10^3–10^4-fold more tightly than tRNA. Binding constants are on the order of 10 μM^{-1} and are only weakly dependent on K^+ concentration ($\partial \log K/\partial \log [K^+] = -1.4$) or temperature. Binding requires multivalent cations; Mg^{2+} is taken up into the complex with an affinity of ~ 3 mM^{-1}. Other multivalent cations tested, Ca^{2+} and $Co(NH_3)_6^{3+}$, promote binding nearly as well. The pH dependence of binding is a bell-shaped curve with a maximum near neutral pH, but the entire curve is shifted to higher pH for the smaller of two RNA fragments tested. This result suggests that the smaller fragment favors a conformation stabilizing protonated forms of the RNA recognition site and is potentially relevant to a hypothesis that this rRNA region undergoes an ordered series of conformational changes during the ribosome cycle.

The discovery of ribozymes (Cech & Bass, 1986) has encouraged speculation that ribosomal RNAs are major contributors to the substrate recognition and catalytic activities of the ribosome. Several kinds of evidence support of this view: ribosomal RNAs are intimately involved in mRNA and tRNA binding and subunit association (Zimmermann et al., 1979; Burma et al., 1983; Jacob et al., 1987); antibiotics which block

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