Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (12 pages). Ordering information is given on any current masthead page.

References

Erter, H., and Horner, L. (1962), *J. Chromatogr. 7,* 269.
Feder, J., Aufderheide, N., and Wildi, B. S. (1976), *Experientia Suppl. 26,* 41.
Feder, J., Brougham, L. R., and Wildi, B. S. (1974), *Biochemistry 13,* 1186.
Feder, J., and Schuck, J. M. (1970), *Biochemistry 9,* 2784.
Gante, J., and Lautsch, W. (1964), *Chem. Ber. 97,* 989.
Goldschmidt, S., and Wick, M. (1951), *Justus Liebigs Ann. Chem. 575,* 217.
Harris, E. D., and Krane, S. M. (1974), *N. Engl. J. Med. 291,* 557, 605, 652.
Henderson, P. J. F. (1972), *Biochem. J. 127,* 321.
Johnson, T. B., and Hemingway, E. H. (1916), *J. Am. Chem. Soc. 38,* 1550.
Kester, W. R., and Matthews, B. W. (1977), *Biochemistry 16,* 2506.
Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H., Fujimoto, K., and Umezawa, S. (1975), *Arch. Biochem. Biophys. 171,* 727.
Latt, S. A., Homquist, B., and Vallee, B. L. (1969), *Biochem. Biophys. Res. Commun. 37,* 333.
Morihara, K. (1967), *Biochem. Biophys. Res. Commun. 26,* 656.
Morihara, K., and Oka, T. (1968), *Biochem. Biophys. Res. Commun. 30,* 625.
Morihara, K., and Tsuzuki, H. (1970), *Eur. J. Biochem. 15,* 374.
Morihara, K., and Tsuzuki, H. (1971), *Arch. Biochem. Biophys. 146,* 291.
Morihara, K., Tsuzuki, H., and Oka, T. (1968), *Arch. Biochem. Biophys. 123,* 572.
Peach, M. J. (1977), *Physiol. Rev. 57,* 313.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27,* 157.
Suda, H., Aoyagi, T., Takeuchi, T., and Umezawa, H. (1973), *J. Antibiot. 26,* 621.
Walsh, K. A., Burstein, Y., and Pangburn, M. K. (1974), *Methods Enzymol. 34,* 435.
Weaver, L. H., Kester, W. R., and Matthews, B. W. (1977), *J. Mol. Biol. 114,* 119.

Cobalt Exchange in Horse Liver Alcohol Dehydrogenase†

Arthur J. Sytkowski‡ and Bert L. Vallee*

ABSTRACT: The preparation of metal hybrid species of horse liver alcohol dehydrogenase is made possible by the development of carefully delineated systems of metal ⇆ metal exchange employing equilibrium dialysis. The conditions which are optimal for the site-specific replacement of the catalytic and/or noncatalytic zinc atoms of the native enzyme by cobalt are not identical with those which are utilized for substitution with ^{65}Zn. Thus, while certain ^{65}Zn hybrids can be prepared by exploiting the differential effects of buffer anions, the cobalt hybrids are generated by critical adjustments in the pH of the dialysate. Factors which may determine the mechanism of metal replacement reactions include acid-assisted, ligand-assisted, and metal-assisted dechelation, steric restriction, and ligand denticity as well as physicochemical properties of the enzyme itself. The spectral characteristics of the catalytic and noncatalytic cobalt atoms reflect both the geometry of the coordination complexes and the nature of the ligands and serve as sensitive probes of these loci in the enzyme.

The role(s) of the intrinsic metal atom(s) of LADH in its catalytic mechanism are presently unknown. Thus far, studies of the kinetics and reaction mechanisms of transition metal model complexes have yielded little information pertaining to the manner in which their chemical properties relate to those of the enzyme·metal coordination complex or to the interactions between the metal atom and the ligands of the enzyme.

We have recently identified the catalytic (C) and noncatalytic (N) metal atoms of horse liver alcohol dehydrogenase [$(LADH)Zn_2Zn_2$] utilizing the differential properties of cobalt-substituted derivatives.[1] Such identification requires the preparation of a series of hybrid enzymes in which the extent and site-specificity of metal replacement are delineated precisely (Sytkowski & Vallee, 1975, 1976). The chemical properties characteristic of the respective metal binding sites become apparent as a consequence.

The present study describes the characteristics of metal exchange in LADH which constitute the basis of the preparation of these hybrid enzymes. Importantly, the conditions

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received March 16, 1978.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health, of the Department of Health, Education and Welfare.

‡ Recipient of Special Fellowship GM 57036 of the National Institute of General Medical Sciences.

[1] Abbreviations used: $[(LADH)Zn_2Zn_2]$ or LADH, native horse liver alcohol dehydrogenase; CD, circular dichroism; EPR, electron paramagnetic resonance. In order to differentiate and clarify presentation, the first pair of exchangeable metal (M) atoms in the standard formulation is designated the "N" (noncatalytic) and the second the "C" (catalytic) pair, i.e., $[(LADH)N_2C_2]$. Hence, Zn and Co represent the (N) pair in $[(LADH)Zn_2M_2]$ and $[(LADH)Co_2M_2]$, while they are the (C) pair in $[(LADH)M_2Zn_2]$ and $[(LADH)M_2Co_2]$.

0006-2960/78/0417-2850$01.00/0

required for metal exchange in the enzyme and the site specificity of this exchange depend upon both the nature of the entering atom as well as that of the enzyme-bound metal atom which is being replaced, thus reflecting exchange mechanisms unique to the reactant metal atoms and their ligands. In addition, the spectral properties of the resultant cobalt derivatives further elucidate the metal binding sites of the enzyme.

Methods

Horse liver alcohol dehydrogenase (LADH) (Boehringer Mannheim Corp.) was obtained as a suspension of crystals in 0.02 M potassium phosphate, 10% ethanol, pH 7. Concentrated solutions of enzyme were prepared by dissolving the crystals in 0.1 M sodium phosphate, pH 7.5, and dialyzing against 100 volumes of this buffer for 12 h at 4 °C followed by two 12-h dialyses against 100 volumes each of 0.1 M Tris–acetate or Tris-HCl, pH 7.5, 4 °C. Enzyme concentrations were determined spectrophotometrically, $E_{280}^{0.1\%} = 0.43$ (Drum & Vallee, 1970a). The specific enzymatic activities of various samples of the enzyme were similar (13.0–15.0 ΔA_{340} min^{-1} mg^{-1}) (Drum et al., 1969a). They contained 3.6–4.1 g-atoms of zinc/mol of LADH as measured by atomic absorption spectrometry (Fuwa et al., 1964).

^{65}Zn-labeled LADH was prepared by a modification of conditions for equilibrium dialysis described previously (Drum et al., 1969b) (see Results); ^{65}Zn (sp act. 3–7 Ci/g) was obtained from New England Nuclear, and radioactivity was determined by γ emission spectrometry (Model 1185, Searle Analytic, Inc.).

Cobalt-substituted LADH was prepared by dialysis of aliquots of native LADH and ^{65}Zn-labeled LADH, 8.4×10^{-5} M, against 50 volumes of 0.2 M $CoCl_2$, pH 5.9 or 5.4, 4 °C. The dialysate was gassed constantly with oxygen-free N_2 (Union Carbide Corp., Linde Div.). Metal exchange was terminated and excess cobalt removed by dialysis against several changes of metal-free 0.2 M Tris–acetate, pH 7.5. Cobalt was measured by atomic absorption spectrometry.

$ZnSO_4 \cdot 7H_2O$ and $CoCl_2 \cdot 6H_2O$ were Johnson-Matthey "Specpure" compounds (Jarrell-Ash Div., Fisher Scientific Co.). All other metal-free reagents, solutions, glassware, and dialysis tubing were prepared as previously described (Thiers, 1957; Drum, 1967).

Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer equipped with 0–0.1 and 0–1.0 absorbance slide wires using quartz sample cells of 1–10-mm pathlength. Circular dichroism (CD) was determined with a Cary 61 spectropolarimeter using quartz sample cells of 0.1–10-mm pathlength. Units of molar ellipticity, $[\theta]$, are deg cm^2 $dmol^{-1}$. The sample compartments of both instruments were purged continuously with N_2 to prevent oxidation of the cobalt enzymes. Sample cells were rendered metal-free by soaking for 12 h in a 1:1 solution of concentrated HNO_3 and H_2SO_4 followed by extensive rinsing with metal-free distilled H_2O. Electron paramagnetic resonance (EPR) spectra were recorded at 4 K using a Varian E9 EPR spectrometer fitted with an Air Products Helitran apparatus; temperature was measured with a Fe doped Au–Chromel thermocouple located adjacent to the sample. All spectra were determined in 0.2 M Tris–acetate, pH 7.5, unless otherwise noted.

Results

All four zinc atoms of native horse liver alcohol dehydrogenase, [(LADH)Zn_2Zn_2], exchange fully when dialyzed against $^{65}Zn^{2+}$ in 0.1 M sodium phosphate, pH 5.5, 4 °C, whereas only the two noncatalytic zinc atoms exchange in 0.1 M sodium acetate (Drum et al., 1967, 1969b; Sytkowski & Vallee, 1976). Utilizing these conditions, native [(LADH)Zn_2Zn_2], 1.9×10^{-4} M, is dialyzed against 1.2×10^{-4} M $^{65}Zn^{2+}$ in 0.1 M sodium phosphate or 0.1 M sodium acetate, pH 5.5, 4 °C, for 48 h, until equilibrium is reached resulting in [(LADH)$^{65}Zn_2{}^{65}Zn_2$] with both the noncatalytic and catalytic pairs of zinc atoms exchanged and [(LADH)$^{65}Zn_2Zn_2$], with only the noncatalytic pair exchanged. [(LADH)-$^{65}Zn_2{}^{65}Zn_2$] is then dialyzed against Zn^{2+}, 1.2×10^{-4} M, 0.1 M sodium acetate, pH 5.5, to replace the noncatalytic pair of ^{65}Zn atoms with Zn, resulting in [(LADH)$Zn_2{}^{65}Zn_2$] where ^{65}Zn remains only at the catalytic sites. The metal content and specific enzymatic activities, $\Delta A_{340} = 14$ min^{-1} mg^{-1}, of all three ^{65}Zn-labeled LADH derivatives are identical with those of the native enzyme. The enzymes are stable for 2–3 months at 4 °C in 0.1 M Tris–acetate or Tris-HCl, pH 7.0, and do not lose activity. Importantly, these derivatives retain their characteristic exchange properties. Thus, in 0.1 M sodium acetate, pH 5.5, *all* of the (N) ^{65}Zn of [(LADH)$^{65}Zn_2Zn_2$] but *none* of the (C) ^{65}Zn of [(LADH)$Zn_2{}^{65}Zn_2$] exchanges with Zn^{2+} while only the first (N) pair ^{65}Zn of [(LADH)$^{65}Zn_2{}^{65}Zn_2$] is replaced. In contrast, when dialyzed against 0.1 M sodium phosphate, pH 5.5, all of the ^{65}Zn in all three species exchanges with Zn^{2+}. There is no evidence of intra- or intermolecular exchange of ^{65}Zn during storage. Hence, these ^{65}Zn-labeled species are identical with the native enzyme with respect to all parameters examined. The preparation of these three radiolabeled LADH species in which either the noncatalytic or the catalytic pair of zinc atoms is labeled specifically with ^{65}Zn permits the precise determination of the exchange kinetics of both pairs of zinc atoms in subsequent metal-replacement experiments (see below).

Cobalt Exchange. To study the functional and structural roles of the metal atoms of LADH, its zinc has been replaced by chromophoric and paramagnetic cobalt atoms (Drum & Vallee, 1970b; Young & Wang, 1971; Takahashi & Harvey, 1973; Drott et al., 1974; Shore & Santiago, 1975; Sloan et al., 1975; Harvey & Barry, 1975, 1976) since this allows detailed studies of their environments by various spectroscopic and resonance methods (Vallee & Wacker, 1970). Utilizing [(LADH)$^{65}Zn_2Zn_2$] and [(LADH)$Zn_2{}^{65}Zn_2$] during metal exchange permits the careful monitoring of the kinetics of Co^{2+} incorporation and the determination of the degree of site-specific replacement of the noncatalytic and catalytic pairs of zinc atoms during the preparation of the cobalt-substituted enzyme.

[(LADH)Co_2Co_2], the fully substituted cobalt derivative, was first prepared by Drum & Vallee (1970b) who used 0.2 M Co^{2+} in 0.1 M sodium acetate, pH 5.5. Under these conditions both the (N) *and* the (C) zinc atoms exchange with Co^{2+} despite the presence of acetate as the buffer anion. This contrasts with the $Zn \leftrightarrows {}^{65}Zn$ exchange system in which the catalytic zinc atoms do not exchange with ^{65}Zn in the presence of acetate (Drum et al., 1969b). Thus, the site-specific replacement of zinc by cobalt is *not* simply the result of the differential effect of buffer anions. Instead, it is the alteration of the pH of the dialysate which provides the means for selective cobalt replacement. Aliquots of [(LADH)$^{65}Zn_2Zn_2$] and [(LADH)$Zn_2{}^{65}Zn_2$], 8.5×10^{-5} M, are dialyzed against 0.2 M $CoCl_2$, 0.1 M sodium acetate, 4 °C, at various pHs, and at specified time intervals aliquots of each enzyme are analyzed for their ^{65}Zn content. The replacement of the noncatalytic pair of ^{65}Zn atoms in [(LADH)$^{65}Zn_2Zn_2$] by cobalt is a monotonic, single-rate process; the rate is related inversely to pH from 6.5 to 5.3 (Figure 1A). In contrast, under these conditions the (C) ^{65}Zn atoms of [(LADH)$Zn_2{}^{65}Zn_2$] do not exchange with cobalt at pH 5.9 or above. However, when the pH is lowered to

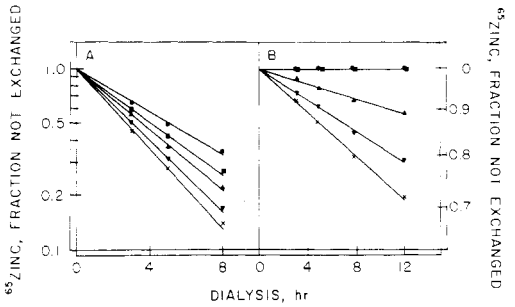


FIGURE 1: pH dependence of cobalt exchange in horse liver alcohol dehydrogenase. Aliquots of [(LADH)$^{65}Zn_2Zn_2$] (panel A) and [(LADH)-$Zn_2{}^{65}Zn_2$] (panel B), 8.5×10^{-5} M, were dialyzed against 0.2 M $CoCl_2$, 0.1 M sodium acetate, 4 °C; pH 6.5 (●), 5.9 (■), 5.7 (▲), 5.5 (▼), 5.3 (X).

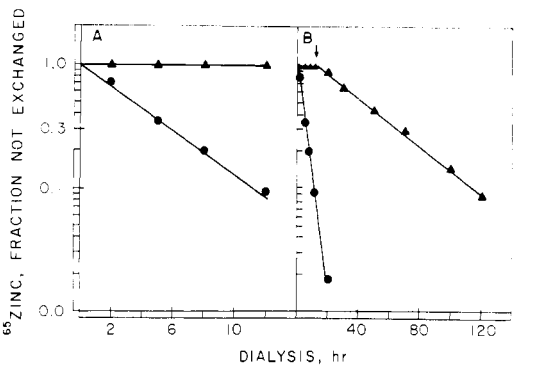


FIGURE 2: Preparation of [(LADH)Co_2Zn_2] (panel A) and [(LADH)-Co_2Co_2] (panel B). Aliquots of [(LADH)$^{65}Zn_2Zn_2$] (●), [(LADH)-$Zn_2{}^{65}Zn_2$] (▲), and native enzyme, 8.4×10^{-5} M, were dialyzed against 0.2 M $CoCl_2$, 0.1 M sodium acetate, pH 5.9, 4 °C. After 12 h the dialysate was discarded and replaced by 0.2 M $CoCl_2$, 0.1 M sodium acetate, pH 5.4, 4 °C.

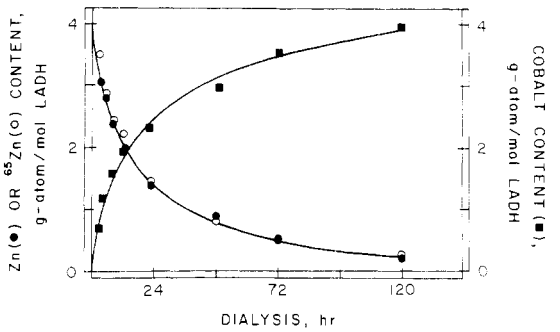


FIGURE 3: ^{65}Zn loss as a measure of zinc ⇄ cobalt exchange in horse liver alcohol dehydrogenase. During cobalt exchange, aliquots of ^{65}Zn-labeled enzyme were analyzed for ^{65}Zn (○) by γ emission spectrometry and aliquots of native enzyme were analyzed for zinc (●) and cobalt (■) content by atomic absorption spectrometry.

pH 5.7 or below, i.e., by as little as 0.2 unit, the rate of exchange of the (C) atoms is then also related inversely to pH (Figure 1B). Attempts to replace zinc with cobalt below pH 5.3 result in denaturation and precipitation of the enzyme. Thus, selective, site-specific replacement of only the (N) zinc atoms of [(LADH)Zn_2Zn_2], i.e., to prepare [(LADH)-Co_2Zn_2], is accomplished by dialysis at pH 5.9 or above, whereas exchange of the (N) and (C) zinc atoms with cobalt occurs at pH 5.7. Therefore, [(LADH)Co_2Co_2] can be prepared either by successive exposure of the zinc enzyme to pH 5.9 followed by a decrease to pH 5.7 (and below) *or* by initial exposure to pH 5.7 or below.

[(LADH)Co_2Zn_2] and [(LADH)$Co_2{}^{65}Zn_2$] were prepared by dialysis of 3 mL each of [(LADH)$^{65}Zn_2Zn_2$] and [(LADH)$Zn_2{}^{65}Zn_2$] and 20 mL of native [(LADH)Zn_2Zn_2], all 8.4×10^{-5} M, in separate dialysis sacs against 1000 mL of 0.2 M $CoCl_2$, 0.1 M sodium acetate, pH 5.9, 4 °C, under 1 atm of N_2. Aliquots were removed from each sac at various times and dialyzed against 0.2 M Tris–acetate, pH 7.5, to terminate exchange and to remove excess cobalt. The radiolabeled enzymes were analyzed for ^{65}Zn, and the native enzyme was examined for its Zn and Co contents, specific enzymatic activity, and spectral characteristics. During the initial 12 h of dialysis at pH 5.9 more than 90% of the noncatalytic ^{65}Zn of [(LADH)$^{65}Zn_2Zn_2$], but none of the catalytic ^{65}Zn of [(LADH)$Zn_2{}^{65}Zn_2$] exchanges with Co^{2+} (Figure 2A). The resultant enzymes, [(LADH)Co_2Zn_2] and [(LADH)-$Co_2{}^{65}Zn_2$], contain 1.9 g-atoms of Co/mol of enzyme at the noncatalytic sites and 2.0 g-atoms of Zn or ^{65}Zn/mol of enzyme at the catalytic sites and have a specific activity ΔA_{340} = 14 min^{-1} mg^{-1}, identical with that of the native enzyme [(LADH)Zn_2Zn_2]. At 12 h, a portion of [(LADH)Co_2Zn_2] was dialyzed against several changes of 0.2 M Tris–acetate, pH 7.5, to terminate the exchange and was stored at 4 °C under N_2. The exchange experiment was continued by discarding the pH 5.9, 0.2 M $CoCl_2$ dialysate and replacing it with 0.2 M $CoCl_2$, 0.1 M sodium acetate, pH 5.4. Upon lowering the pH, the (C) zinc atoms begin to exchange (Figure 2B). The dialysis was continued for 120 h with two buffer changes resulting in greater than 95% replacement of both pairs of Zn atoms by cobalt to yield [(LADH)Co_2Co_2], containing 3.9 g-atoms of cobalt and 0.2 g-atoms of Zn/mol of enzyme and exhibiting a specific enzymatic activity of 9 min^{-1} mg^{-1}, 64% of that of the native enzyme. This lower specific activity of [(LADH)Co_2Co_2] is consistent with previous reports of that of LADH completely substituted with cobalt (Drum & Vallee, 1970b; Young & Wang, 1971; Takahashi & Harvey, 1973; Sloan et al., 1975) and reflects the replacement of zinc by cobalt at the second catalytic pair of metal binding sites (Sytkowski & Vallee, 1976).

The accuracy and reliability of ^{65}Zn loss as a measure of Co incorporation in the enzyme were confirmed by metal analyses of aliquots of the enzyme removed during dialysis (Figure 3). The loss of total ^{65}Zn, i.e., the Σ(catalytic ^{65}Zn + noncatalytic ^{65}Zn) is accompanied by a corresponding decrease in total Zn content (g-atom/mol of LADH) measured by atomic absorption spectrometry and an increase in Co content. The Σ[Zn + Co] in the various samples ranges from 3.7 to 4.1 g-atoms/mol of enzyme. Thus, under the above conditions, loss of ^{65}Zn from either the noncatalytic or catalytic sites is a reliable parameter of the site specificity of replacement of both types of zinc atoms by cobalt.

In contrast to the above results, all efforts to prepare a hybrid enzyme with Zn at the noncatalytic sites and Co only at the catalytic sites, "[LADH)Zn_2Co_2]," have been unsuccessful thus far. Neither careful selection of pH nor changes in buffer anions have resulted in this selective exchange of the (N) Co atoms of [(LADH)Co_2Co_2] with Zn. For example, dialysis of 5×10^{-5} M [(LADH)Co_2Co_2] against 0.1 mM $ZnSO_4$, 0.2 M Tris–acetate, pH 7.5, 4 °C, conditions under which the intrinsic *Zn* would not be displaced from the enzyme results in *loss of Co* from both pairs of sites, albeit at two apparent first-order rates (Figure 4); this may indicate an exchange mechanism different from those operative in the preparation of the other metal-substituted derivatives (see Discussion).

Spectral Properties. The coordination complexes of the (N) and (C) cobalt atoms in LADH are optically active and, hence, exhibit circular dichroic extrema (Figure 5) corresponding to

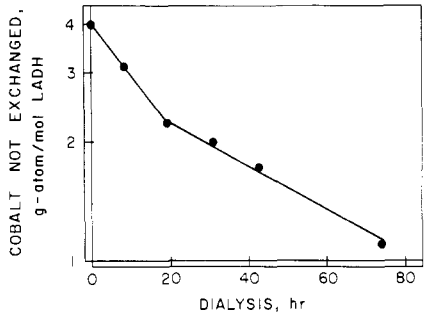


FIGURE 4: Zinc exchange in [(LADH)Co_2Co_2]. Enzyme, 5×10^{-5} M; 0.1 mM $ZnSO_4$; 0.2 M Tris-acetate, pH 7.5, 4 °C. Cobalt content (●) of aliquots determined by atomic absorption spectrometry.

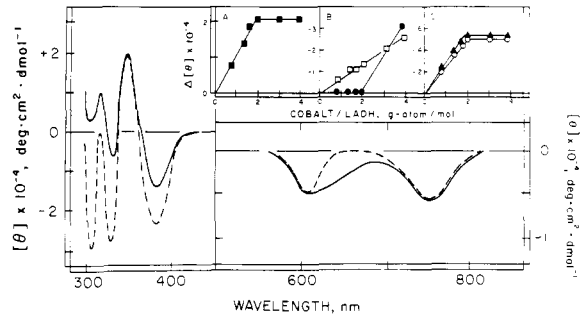


FIGURE 5: Circular dichroic spectra of [(LADH)Co_2Zn_2] (—) and [(LADH)Co_2Co_2] (- - -). Insets: Circular dichroic extrema of LADH vs. cobalt content during cobalt exchange; (inset A) 355 nm (■); (inset B) 305 nm (●) and 387 nm (□); (inset C) 600 nm (○) and 745 nm (▲); 0.2 M Tris-acetate (pH 7.5), 4 °C.

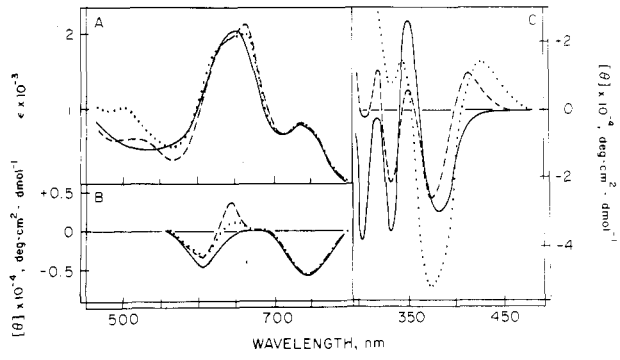


FIGURE 6: Absorption (panel A) and circular dichroic (panels B and C) spectra of [(LADH)Co_2Co_2] in the absence (—) or presence of pyridine (- - -)or 2,5-dimethylpyrazine (• • •). Enzyme, 0.1 mM; pyridine or 2,5-dimethylpyrazine, 88 mM; 0.2 M Tris-acetate, pH 7.5, 4 °C.

the 340, 655, and 740 nm absorbance maxima described previously (Sytkowski & Vallee, 1976). The absorbance at 340 nm is reflected in a series of positive and negative ellipticity extrema between 300 and 400 nm. For [(LADH)Co_2Zn_2] the molar ellipticities, $[\theta]$, in deg cm^2 dmol^{-1} are λ_{323} (+10 500), λ_{335} (−7000), λ_{355} (+20 500), λ_{387} (−15 000), and for [(LADH)Co_2Co_2] they are λ_{305} (−31 000), λ_{323} (0), λ_{335} (−28 500), λ_{355} (+20 500), λ_{387} (−30 000). Although the spectral absorption band with a maximum at 340 nm does not allow for the discrimination between the contributions of the catalytic and noncatalytic cobalt atoms, the corresponding circular dichroic extrema do permit such differentiation. Thus, the positive ellipticity at 355 nm reflects the presence of cobalt *only* at the noncatalytic sites (Figure 5, inset A). In contrast, the ellipticity at 387 nm reflects the presence of Co at *both* pairs of sites (Figure 5, inset B). Finally, the ellipticity at 305 nm appears *only* when cobalt occupies the catalytic sites (Figure 5, inset B). The absorbance maxima of both enzymes at 655 and 740 nm are optically active as is apparent from circular dichroic extrema at 600, 640 (sh) and 745 nm with molar ellipticities, $[\theta]$, in deg cm^2 dmol^{-1} for [(LADH)-Co_2Zn_2] of λ_{600} (−5000), λ_{640} (−3500), λ_{745} (−6000) and for [(LADH)Co_2Co_2] of λ_{600} (−5000), λ_{640} (0), λ_{745} (−6000). The extrema at 600 and 745 nm both reflect only the noncatalytic cobalt atoms (Figure 5, inset C); when Co enters the catalytic sites, the shoulder at 640 nm disappears. Together with the absorption spectra, the circular dichroic spectra of these two cobalt enzyme species serve as probes of the catalytic and noncatalytic metal binding sites. The effects of pyridine and 2,5-dimethylpyrazine, both inhibitors of LADH catalyzed ethanol oxidation, on the absorption and circular dichroic spectra of [(LADH)Co_2Co_2] support this conclusion (Figure 6). Pyridine is competitive with both NAD^+ and ethanol (Theorell et al., 1969) and induces a bathochromic, hyperchromic shift in the maximum at 655 nm (ϵ 2050) to 672 nm, increasing the molar absorptivity to ϵ 2170 while a shoulder at 634–645 nm remains. In addition, pyridine generates a new maximum at 520 nm (ϵ 600) (Figure 6, panel A). The circular dichroic spectrum displays a new positive extremum at 638 nm with a molar ellipticity $[\theta]_{638}$ = +3800 deg cm^2 dmol^{-1}. The CD spectrum in the near-UV differs strikingly, with a new positive extremum at 415 nm, $[\theta]_{415}$ = +10 900 deg cm^2 dmol^{-1} and significant shifts and/or alterations in molar ellipticities of the other extrema (Figure 6, panel C). These effects of pyridine on the spectra of [(LADH)Co_2Co_2] are distinguished easily from those of 2,5-dimethylpyrazine which, in contrast to pyridine, manifests competitive inhibition and stimulation type of inhibition toward NAD^+ and ethanol (Theorell et al., 1969). In the visible region the absorption and CD spectra of [(LADH)Co_2Co_2] in the presence of the two inhibitors are somewhat similar (Figure 6, panels A and B), although in the presence of 2,5-dimethylpyrazine the new absorption maximum is located at 500 nm (ϵ 1000). However, there are marked differences in the near-UV of the CD region (Figure 6C). Such spectral perturbations may allow the mapping of the topology of the active center with respect to the sites of interaction of inhibitors, coenzyme, or substrates (Alter et al., 1976).

The electron paramagnetic resonance (EPR) spectra of both [(LADH)Co_2Zn_2] and [(LADH)Co_2Co_2] exhibit a low field component centered at $g' \cong 6$; resonances at higher field are less well resolved (Figure 7). The $g' \cong 6$ components of both spectra exhibit additional detail consisting of 6–8 hyperfine lines (Figure 7, insets A and B). Comparison of these spectra with those of known model complexes does not allow definitive conclusions regarding the specific coordination geometries giving rise to these spectra, but they do suggest that the environments both of the noncatalytic and catalytic metal atoms are distorted (Kennedy et al., 1972). In contrast to the effects of coenzyme and substrate analogues and inhibitors on the absorption and CD spectra of cobalt-substituted LADH (see above) thus far little or no effect on the cobalt EPR spectra has been observed. The chelating agent 1,10-phenanthroline is the single exception. It removes the catalytic Co atoms from [(LADH)Co_2Co_2] (Sytkowski & Vallee 1976, 1978) and thereby alters the cobalt EPR spectrum of this derivative markedly (Figure 8). As expected, 1,10-phenanthroline does not affect the EPR spectrum of [(LADH)Co_2Zn_2].

Discussion

The preparation of a series of hybrid metallodehydrogenases in which the respective zinc atom pairs of the native enzyme

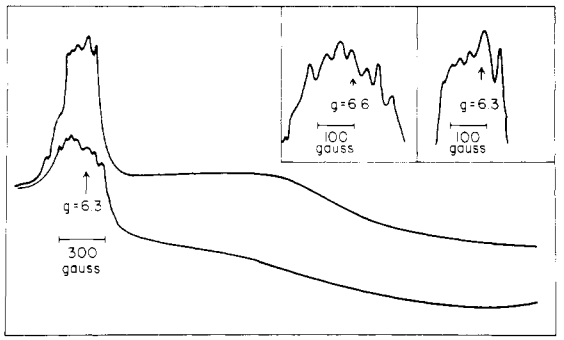


FIGURE 7: Electron paramagnetic resonance (EPR) spectra of [(LADH)Co_2Zn_2] (lower trace, left inset) and [(LADH)Co_2Co_2] (upper trace, right inset). Enzyme, 1.4×10^{-4} M; 0.2 M Tris-acetate (pH 7.5). Power setting 60 mW, modulation frequency 100 kHz, microwave frequency 9.39 kHz, receiver gain 6000. See Results.

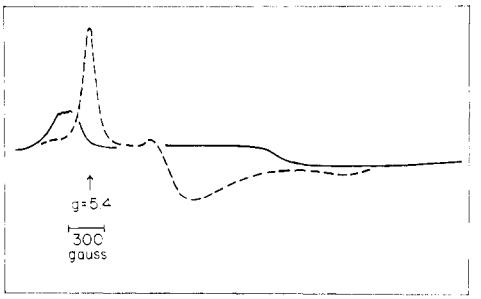


FIGURE 8: EPR spectrum of [(LADH)Co_2Co_2] in the absence (—) and presence (- - -) of 1,10-phenanthroline. Enzyme, 1.4×10^{-4} M; Tris-acetate 0.2 M, pH 7.5. Receiver gain 2000; other settings as in Figure 6. Note marked perturbation and new g' value ($g' = 5.4$) in the presence of 1 mM 1,10-phenanthroline.

are specifically replaced by ^{65}Zn or cobalt has allowed the identification and spectroscopic characterization of the catalytic and noncatalytic metal atoms of horse liver alcohol dehydrogenase. The substitution of chromophoric metal atoms for zinc has been accomplished in other enzymes, e.g., carboxypeptidase (Coleman & Vallee 1960, 1961), alkaline phosphatase (Simpson & Vallee 1968; Anderson et al., 1976), carbonic anhydrase (Lindskog, 1970), thermolysin (Holmquist & Vallee, 1974), leucine amino peptidase (Thompson & Carpenter, 1976), and others (Vallee & Wacker, 1970) by removing the native metal atoms with chelating agents resulting in an inactive apoenzyme followed by introduction of a different metal atom added directly to the apoenzyme. However, it has not proven possible to prepare an apoenzyme of LADH which can be reactivated directly by metal addition in this manner. Hence, the native zinc atoms of LADH have been replaced utilizing various methods of equilibrium dialysis against other metal atoms, an approach which has recently been applied also to yeast alcohol dehydrogenase (Sytkowski, 1978).

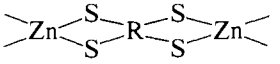
Site Specificity of Cobalt Replacement. The differential replacement of the (C) and (N) zinc atoms takes advantage of the chemical properties inherent in the coordination complexes at the respective binding sites which govern the different conditions and rates of exchange for the two pairs of atoms. Basically, the conditions of $Zn \rightleftharpoons {}^{65}Zn$ exchange employed here are those of Drum et al. (1969b) who first succeeded in preparing [(LADH)$^{65}Zn_2Zn_2$] and [(LADH)$^{65}Zn_2{}^{65}Zn_2$]. These methods of ^{65}Zn replacement have been extended by exchange of [(LADH)$^{65}Zn_2{}^{65}Zn_2$] against stable zinc to yield [(LADH)$Zn_2{}^{65}Zn_2$]. The preparation of these hybrid enzymes, [(LADH)$^{65}Zn_2Zn_2$] and [(LADH)$Zn_2{}^{65}Zn_2$], radiolabeled at the noncatalytic and catalytic sites, respectively, is the critical step which allows the precise delineation of the site-specific replacement by cobalt. Importantly, conditions which prove optimal for cobalt replacement are *not identical* with those for $Zn \rightleftarrows {}^{65}Zn$ exchange, and, thus, the use of the latter conditions, specifically at pH 5.5, precludes site-specific substitution of cobalt for zinc. Thus, dialysis of native [(LADH)Zn_2Zn_2] against 0.1 M sodium acetate, pH 5.5, containing 1×10^{-4} M $^{65}Zn^{2+}$ results in the exchange of only the first, noncatalytic pair of Zn atoms, whereas dialysis of this enzyme against the same 0.1 M sodium acetate buffer at pH 5.5 containing 0.2 M Co^{2+} instead of Zn^{2+} results in the exchange of *both* the noncatalytic and catalytic pairs of Zn atoms (see Results).

Metal Exchange in Model Systems. The mechanisms of metal $\rightleftarrows$ metal exchange of the catalytic and noncatalytic zinc atoms of LADH are unknown and cannot be predicted directly based on data derived from model systems, since the binding and exchanging of zinc and cobalt in analogous sulfur donor systems have not yet been studied. Most of the information available presently has been obtained for both different donor atoms and metal pairs. Hence, it can at best only partially elucidate factors which may pertain to enzymatic systems. Metal $\rightleftarrows$ metal exchange in such uni- and multidentate systems demonstrates that it is the nature of the intermediate mixed complex which determines the rate of exchange (Wilkins, 1974; Margerum et al., 1978). Moreover, in those multidentate systems which have been examined the prevailing stepwise, ordered reaction mechanisms are subject to a number of variables.

Thus, in the complex ML, the transfer of coordinated ligand(s) (L) from the metal atom (M) to the incoming metal atom (M') may be assisted by M' itself.

$$ML + M' \rightarrow MLM' \rightarrow M + LM' \qquad (1)$$

In unidentate systems such metal-assisted removal reactions are apparently due to the substitution of one electron-deficient species for another (electrophilic substitution reactions), while in multidentate systems (metal-assisted dechelation) the formation of binuclear complexes is thought to be of import as part of the overall reaction mechanism (Wilkins, 1974). The possible formation of binuclear complexes in the metal-assisted dechelation of the (N) and (C) zinc atoms of LADH is of particular interest in the light of the propensity of zinc (Leussing & Tischer, 1961) and cobalt (Jicha & Busch, 1962) to form bridged dimercaptides of the form

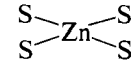


Since the noncatalytic metal pair of the enzyme is coordinated to four sulfur ligands (Cys-97, -100, -103, -111) while the catalytic pair is coordinated to only two (Cys-46 and -174) and the N-3 of His-67 (Brändén et al., 1975), the more rapid rate of exchange (metal-assisted dechelation) of the noncatalytic metal atoms (see Results) may be due, in part, to the greater probability of the formation of such binuclear complexes at these sites.

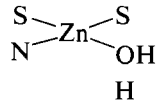
Additional studies on replacement of the Zn atoms of LADH by cadmium (A. J. Sytkowski & B. L. Vallee, in preparation) or by other metal atoms (A. J. Sytkowski & B. L. Vallee, unpublished observations) reveal that for each element to be introduced and for each pair of elements replacing each other the conditions for exchange must be established specifically. The observations leading to these conclusions likely reflect the characteristics of the intermediate formed as

a function of the enzyme-bound metal, M, and that which displaces it, M′, and their respective roles in the mechanism of exchange.

In addition to metal-assisted dechelation, the removal of ligands from M and the transfer to M′ are influenced by the presence of protons and additional ligands in the environment, i.e., acid-assisted and ligand-assisted dechelation (Wilkins, 1974). Both these factors likely play roles in the metal ⇄ metal exchange of LADH. The earlier studies of Drum (1967) demonstrate that the rate of Zn ⇄ ^{65}Zn exchange relates directly to hydrogen ion concentration. Similarly, the present study demonstrates that pH critically affects Zn ⇄ Co exchange. Protonation of one or several of the ligands of the noncatalytic zinc atoms



or of the catalytic zinc atoms



could disrupt the metal's coordinate bonds and either initiate and/or facilitate the ordered mechanism of dechelation and subsequent replacement by the incoming metal.

The pH dependence of metal exchange in proteins is, of course, complicated further by the possibility that protonation of one or several amino acid residues which are not coordinated directly to the metal atom may result in protein conformational changes which also could be critical to the exchange mechanism. Thus, the pH dependence of metal ⇄ metal exchange in LADH may reflect both acid-assisted dechelation at the metal binding site itself and hydrogen ion dependent physicochemical characteristics of the protein.

There may also be dual effects of other potential ligands present in the system, viz., buffer ions. Thus, such ligands may enter the metal coordination sphere and render a ligand which is already coordinated labile to hydrolysis, resulting in ligand-assisted dechelation (Wilkins, 1974). Further, much as protons may affect the physicochemical properties of proteins in solution, so buffer cations and anions may be critical. This is especially pertinent, since the initial observations of Zn ⇄ ^{65}Zn exchange had shown this exchange to be strictly dependent on the presence of specific concentrations of acetate or phosphate (Drum et al., 1969b), observations which we have confirmed. In this regard, we have also observed that the rates of Zn ⇄ ^{65}Zn and Zn ⇄ Co exchange in LADH depend strongly upon the nature and concentration of the ions present in the order $Cl^- > Br^- >$ acetate$^- > SO_4^{2-}$ and $Li^+ > Na^+$ (A. J. Sytkowski & B. L. Vallee, unpublished observation), remarkably similar to the lyotropic series reported for helix ⇄ coil transitions of collagen and folding ⇄ unfolding transitions of ribonuclease (von Hippel & Schleich, 1969). These effects call for careful attention to both the nature and concentration of buffer ions throughout all studies of metal ⇄ metal exchange in LADH. The mechanisms thus may be functions of metal, acid, and ligand assistance in metal ⇄ metal exchange of the coordination complex itself and of proton and buffer ion effects on specific amino acid residues or general protein structure.

Steric Effects. The stepwise transfer of ligands from M to the incoming M′ is almost certainly influenced by the conformation of the protein at the metal binding site, i.e., the steric freedom or restriction of the ligands and those portions of the polypeptide chains of which they are a part. For example, studies on model chelates reveal that the rate constant for removal of Cu(II) from copper tripeptide complexes of the type Cu(H_{-2} tripeptide) by the nucleophile triethylenetetramine is 200-fold lower for the sterically restricted Cu(H_{-2}Gly·*Leu*·Gly) than for Cu(H_{-2}Gly·*Gly*·Gly) (Hauer et al., 1973). Furthermore, experiments on association of Cu(II) with macrocyclic polyamine ligands demonstrate a striking reduction in the rate constant of association from $k = 10^7$ M^{-1} s^{-1} for the flexible quadridentate ligand *N,N*-bis(2-aminoethyl)-1,3-propanediamine to $k = 2 \times 10^{-2}$ M^{-1} s^{-1} for the rigid hematoporphyrin IX (Cabbiness & Margerum, 1970).

Steric restriction is known to be important not only in such association and dissociation of a metal atom with a ligand but also in the kinetics of replacement of one metal for another in coordination complexes—a metal ⇄ metal exchange system analogous to that in alcohol dehydrogenase. Metal substitutions in ethylenediaminetetraacetatonickelate(II), $[NiEDTA]^{2-}$, reveal that the rate constant of copper(II) of substitution is 20 000-fold faster than the corresponding one for ^{63}Ni(II). The kinetic expression for Cu(II) substitution in this chelate is first order with respect to entering metal and is not acid catalyzed (Bydalek & Margerum, 1961). In marked contrast, substitution of copper(II) in cyclohexylenediaminetetraacetatonickelate(II), $[NiCyDTA]^{2-}$, is zero order with respect to copper and depends markedly on pH. The chelates of EDTA and CyDTA differ in that the iminodiacetate segments of EDTA rotate freely while the cyclohexane ring of CyDTA prevents full rotation of its two iminodiacetate segments (Margerum & Bydalek, 1963). In this instance steric restriction of the ligand CyDTA not only alters the rate of metal substitution but has also changed the mechanism of the substitution itself as reflected in the appearance of pH dependence of the exchange. Thus, the different mechanisms of Cu(II) exchange in $[NiEDTA]^{2-}$ and $[NiCyDTA]^{2-}$ are due to the absence or presence of steric restriction of the ligand.

Ligand Denticity. In addition to such steric considerations, the denticity of the ligand can play an important role in the rate of metal exchange. Replacement of Cu(II) in a series of Ni(II) polyamines of increasing dentate number reveals that the rate of transfer of the ligand to copper increases with increasing denticity (Margerum et al., 1978). This has been interpreted in terms of a transition state favorable to the entry of Cu(II). The metal ⇄ metal exchange system in LADH may reflect increasing denticity, since the more rapidly exchanging, noncatalytic metal atoms are coordinated by four protein ligands, while the more slowly exchanging, catalytic metal atoms are coordinated by only three.

Although the parameters which are pertinent to metal exchange in model systems, i.e., acid, ligand, and metal assistance, steric restriction, denticity, etc., may illustrate the complexity of such exchange processes, the same variables may or may not pertain to metalloproteins in the same manner. In any event, definitive conclusions about the mechanism(s) of metal ⇄ metal exchange in metalloenzymes cannot be based on such studies alone. Clearly, what might be generalized to become conventional wisdom pertaining to metal exchange kinetics as derived from model systems cannot predict what may be expected to occur in a metalloenzyme such as LADH, a system which is inherently more complex. The parameters governing these aspects of the behavior of metalloenzymes are only now being developed.

Spectral Properties. The spectral properties of metalloenzymes can provide valuable information regarding both the nature of the ligands and the geometry of the coordination complexes (Vallee & Wacker, 1970). Both $[(LADH)Co_2Zn_2]$ and $[(LADH)Co_2Co_2]$ exhibit relatively intense absorption

maxima at 340 nm ($\epsilon > 1000$ per Co(II) atom) (Sytkowski & Vallee, 1976). This absorbance almost certainly reflects charge-transfer between cobalt and sulfur ligands at both the catalytic and noncatalytic metal binding sites (Brändén et al., 1975). Such charge-transfer phenomena are well-known from studies of model complexes of zinc and of the transition elements with sulfur ligands such as thioglycolate or cysteine (Leussing, 1958; Garbett et al., 1972). The absorption spectra of several metalloproteins in which the metal atom is coordinated to sulfur ligands all reveal charge-transfer bands ($\epsilon >$ 1000), while these are absent from spectra of cobalt-substituted carboxypeptidase and carbonic anhydrase, enzymes which do not contain metal mercaptides. Notably, the wavelength of this charge transfer band in proteins shifts bathochromically along a series Fe(III) > Cu(II) > Co(II) > Fe(II) > Cd(II) > Zn(II), reflecting increasing electron affinity of the cation (Garbett et al., 1972). Indeed, it was the striking similarity between the charge-transfer band of cadmium-substituted liver alcohol dehydrogenase and that found in cadmium metallothionein, a protein in which this metal is known to be bound to cysteine, which first led to the proposal that the metal atoms of the dehydrogenase also might be coordinated to sulfur (Druyan & Vallee, 1962, Ulmer & Vallee, 1965; Drum & Vallee, 1970b). An analogous absorption band, λ_{340}, in cobalt-substituted yeast alcohol dehydrogenase [(YADH)Co_4] strongly suggests that the active site metal atoms in this enzyme are also coordinated to sulfur ligands (Sytkowski, 1978).

The visible and near-infrared regions of the absorption spectrum can reflect details of the coordination geometry of cobalt complexes. Hexaaquocobalt(II), $[Co(H_2O)_6]^{2+}$, the state of coordination of the cobalt ion in aqueous solution is a regular octahedral complex characterized by an absorption maximum at 510 nm of very low absorptivity ($\epsilon \sim 5$). On the other hand, simple tetrahedral cobalt(II) complexes such as $[CoCl_4]^{2-}$ exhibit maxima in the 600–750-nm region with far greater absorptivities λ_{685} (ϵ 700). The intensities per cobalt atom and positions of the absorbance maxima of [(LADH)-Co_2Zn_2] and [(LADH)Co_2Co_2] are very similar to those found in tetrahedral model complexes of somewhat distorted geometry, suggesting analogous cobalt coordination in the enzyme (Foster et al., 1970). Such a conclusion is strongly supported both by the earlier finding of an absorption maximum for [(LADH)Co_2Co_2] in the near-infrared region, 1000–1800 nm (Drum & Vallee, 1970b), known to be characteristic of tetrahedral cobalt complexes (Goodgame & Goodgame, 1965) and by subsequent x-ray crystallographic studies of native LADH (Brändén et al., 1975) which demonstrated tetradentate coordination of both the catalytic and noncatalytic zinc atoms of the enzyme.

A series of tetrahedral mercaptide complexes of first row transition elements has been synthesized recently (Coucouvanis et al., 1975). The dithiophenolatodithiosquaratocobaltate(II), $[(Co(SPh)_2dts]^{2-}$, and tetrathiophenolatocobaltate(II), $[Co(SPh)_4]^{2-}$, are of special pertinence to LADH. Both of these tetrahedral mercaptide complexes exhibit absorption maxima both in the 600–800 nm region and the near-infrared. The remarkable similarity of the absorption spectra of these complexes with those of the cobalt-substituted alcohol dehydrogenases provides strong evidence for the existence of tetrahedral coordination geometry at the noncatalytic and catalytic sites of the enzyme.

The metal $\rightleftharpoons$ metal exchange properties of LADH presented here reflect the chemical differences between the catalytic and noncatalytic metal binding sites of the enzyme, while the studies performed illustrate a general approach to the exploration of multichain enzymes containing more than one metal atom. Moreover, the differentiation of these metal binding sites forms the basis for studies of metal–protein and metal–ligand complexes and for the elucidation of both the functional and structural roles of metal atoms in enzymes.

Acknowledgment

We wish to thank Dr. Dennis Sardella for invaluable assistance in obtaining the EPR spectra.

References

Alter, G. M., Klyosov, A. A., & Johansen, J. T. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1645.

Anderson, R. A., Kennedy, F. S., & Vallee, B. L. (1976) *Biochemistry 15,* 3710.

Brändén, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11A,* 104.

Bydalek, T. J., & Margerum, D. W. (1961) *J. Am. Chem. Soc. 83,* 4326.

Cabbiness, D. K., & Margerum, D. W. (1970) *J. Am. Chem. Soc. 92,* 2151.

Coleman, J. E., & Vallee, B. L. (1960) *J. Biol. Chem. 235,* 390.

Coleman, J. E., & Vallee, B. L. (1961) *J. Biol. Chem. 236,* 2244.

Coucouvanis, D., Holah, D. G., & Hollander, F. J. (1975) *Inorg. Chem. 14,* 2657.

Drott, H. R., Santiago, D., & Shore, J. D. (1974) *FEBS Lett. 39,* 21.

Drum, D. E. (1967) Ph.D. Thesis, Massachusetts Institute of Technology.

Drum, D. E., & Vallee, B. L. (1970a) *Biochemistry 9,* 4078.

Drum, D. E., & Vallee, B. L. (1970b) *Biochem. Biophys. Res. Commun. 41,* 33.

Drum, D. E., Harrison, J. H., IV, Li, T.-K., Bethune, J. L., & Vallee, B. L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57,* 1434.

Drum, D. E., Li, T.-K., & Vallee, B. L. (1969a) *Biochemistry 8,* 3783.

Drum, D. E., Li, T.-K., & Vallee, B. L. (1969b) *Biochemistry 8,* 3792.

Druyan, R., & Vallee, B. L. (1962) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 21,* 247.

Foster, M. A., Hill, H. A. O., & Williams, R. J. P. (1970) *Biochem. Soc. Symp. No. 31,* 187–202.

Fuwa, K., Pulido, P., McKay, R., & Vallee, B. L. (1964) *Anal. Chem. 36,* 2407.

Garbett, K., Partridge, G. W., & Williams, R. J. P. (1972) *Bioinorg. Chem. 1,* 309.

Goodgame, D. M. L., & Goodgame, M. (1965) *Inorg. Chem. 4,* 139.

Harvey, R. A., & Barry, A. (1975) *Biochem. Biophys. Res. Commun. 66,* 935.

Harvey, R. A., & Barry, A. (1976) *Biochem. Biophys. Res. Commun. 72,* 886.

Hauer, H., Dukes, G. R., & Margerum, D. W. (1973) *J. Am. Chem. Soc. 95,* 3513.

Holmquist, B., & Vallee, B. L. (1974) *J. Biol. Chem. 249,* 4601.

Jicha, D. C., & Busch, D. H. (1962) *Inorg. Chem. 1,* 872.

Kennedy, F. S., Hill, H. A. O., Kaden, T. A., & Vallee, B. L. (1972) *Biochem. Biophys. Res. Commun. 48,* 1533.

Leussing, D. L. (1958) *J. Am. Chem. Soc. 80,* 4180.

Leussing, D. L., & Tischer, T. N. (1961) *J. Am. Chem. Soc.*

83, 65.
Lindskog, S. (1970) *Struct. Bond. (Berlin) 8,* 153-196.
Margerum, D. W., & Bydalek, T. J. (1963) *Inorg. Chem. 2,* 683.
Margerum, D. W., Cayley, G. R., Weatherburn, B. C., & Pagenkopf, G. K. (1978) *Coord. Chem.* (in press).
Shore, J. D., & Santiago, D. (1975) *J. Biol. Chem. 250,* 2008.
Simpson, R. T., & Vallee, B. L. (1968) *Biochemistry 7,* 4343.
Sloan, D. L., Young, J. M., & Mildvan, A. S. (1975) *Biochemistry 14,* 1998.
Sytkowski, A. J. (1978) *Arch. Biochem. Biophys. 184,* 505.
Sytkowski, A. J., & Vallee, B. L. (1975) *Biochem. Biophys. Res. Commun. 67,* 1488.
Sytkowski, A. J., & Vallee, B.L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 344.
Sytkowski, A. J., & Vallee, B. L. (1978) in *Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E., Eds.) Plenum Press, New York, N.Y. (in press).
Takahashi, M., & Harvey, R. A. (1973) *Biochemistry 12,* 4743.
Theorell, H., Yonetani, T., & Sjöberg, B. (1969) *Acta Chem. Scand. 23,* 255.
Thiers, R. (1957) *Methods Biochem. Anal.* 273-335.
Thompson, G. A., & Carpenter, F. H. (1976) *J. Biol. Chem. 251,* 1618.
Ulmer, D. D., & Vallee, B. L. (1965) *Adv. Enzymol.,* 37-104.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins, 2nd Ed.,* 5.
von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules,* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 417-574, Marcel Dekker, New York, N.Y.
Wilkins, R. G. (1974) *The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes,* Allyn and Bacon, Boston, Mass.
Young, J. M., & Wang, J. H. (1971) *J. Biol. Chem. 246,* 2815.

Purification and Characterization of a Marine Bacterial Collagenase†

Joseph R. Merkel* and Joseph H. Dreisbach‡

ABSTRACT: A true collagenase was isolated from the culture fluid of a marine bacterium which has been designated *Vibrio* B-30 (ATCC 21250). Collagenase production was obtained only in media containing collagen or certain degradation products of collagen. Partial purification on DEAE-cellulose and Sephadex G-200 columns produced active enzyme which was free of nonspecific proteases but which contained two collagenases. The two collagenases have the same apparent molecular size, and evidence is presented to support the theory that one collagenase is derived from the other. *Vibrio* B-30 collagenase appears to be a tetramer with a molecular weight of about 105 000 composed of two different subunits (mol wt 24 000 and 28 000). Some of the properties of the *Vibrio* collagenase are compared with those of *Clostridium histolyticum* collagenase. Molecular weights, subunit structures, specificity and mode of collagen hydrolysis, insensitivity to diisopropyl fluorophosphate and calf serum, and sensitivity to certain metal ion complexing agents and isopropyl alcohol are similar for the collagenases from both organisms. However, *Vibrio* B-30 collagenase and *Clostridium* collagenase differ immunologically and electrophoretically.

We recently reported on the collagenolytic activity of certain marine bacteria (Merkel et al., 1975). Reconstituted, acid-extracted collagen was used to prepare a medium to screen proteolytic marine bacteria for their ability to elaborate collagenolytic enzymes. Approximately 44% of the proteolytic marine isolates were capable of producing collagenases when they were grown in the presence of collagen or degradation products of collagen. One of the most active isolates, designated *Vibrio* B-30,[1] was selected for further studies. The production, purification, and partial characterization of a true collagenase of *Vibrio* B-30 is the subject of this report. Attention is drawn to the similarities and differences between the collagenase produced by *Vibrio* B-30 and that produced by *Clostridium histolyticum* (Keller & Mandl, 1963; Yoshida & Noda, 1965; Lee-Own & Anderson, 1975).

Materials and Methods

Collagenase Production. Two collagen-containing media were used in enzyme production studies and these had the following compositions: (1) CHD½SW contained 2 g of NZ-amine, type HD, hydrolyzed casein (Sheffield Chem. Co.), 400 mL of a 1:3 dilution of acid-extracted, undialyzed calfskin collagen, 20 g of Rila Marine Mix, and distilled water to a total volume of 1 L, adjusted to pH 7.0-7.2; and (2) CHC½SW was similar to CHD½SW except that 40 mL of pancreatin-digested casein (Prescott & Wilkes, 1966) was used in place of the commercial hydrolyzed casein.

Medium was also prepared using commercial gelatin as a substitute for acid-extracted calfskin collagen. The gelatin medium (referred to as HDG½SW) contained 10 g of NZ-amine, type HD hydrolyzed casein, 20 g of gelatin (Fisher Sc.

† From the Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015. *Received September 27, 1977.*

‡ Supported in part by a graduate fellowship from the Jesse Noyes Foundation through the Wetlands Institute of Lehigh University. Part of the data used in the report was abstracted from the doctoral thesis of J.H.D.

[1] Details on the isolation, cultivation, and characterization of this marine bacterium can be obtained from the senior author.

0006-2960/78/0417-2857$01.00/0