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Evidence for Inner-sphere Coordinated Alkoxide Ion Intermediates in the Catalytic Mechanism of Co(II)-Substituted Liver Alcohol Dehydrogenase

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Substitution of certain divalent transition metal ions, *i.e.* Co(II), Ni(II), Cu(II) and Fe(II), for the active site zinc ion of horse liver alcohol dehydrogenase yields catalytically active enzyme derivatives [1–4]. Since catalysis occurs *via* inner-sphere coordination of substrate [3, 5, 6], the ligand-to-metal charge-transfer (LMCT) and d–d electronic transitions of these metal ions are useful spectroscopic probes of the chemical events occurring during catalysis [1, 2, 7]. As will be shown in this report, when reaction is monitored *via* rapid-scanning, stopped-flow (RSSF) UV-visible spectroscopy, the resulting time-resolved spectra (300 nm to 700 nm) provide detailed temporal information about transient intermediates formed during catalysis. We report here evidence derived from RSSF spectroscopy for the existence of ternary enzyme–NAD^+–alkoxide ion intermediates both in the Co(II)E-catalyzed reduction of aldehydes and in the Co(II)E-catalyzed oxidation of alcohols.

Experimental

Site-specifically-substituted Co(II)E was prepared by the method of Maret *et al.* [1]. The preparation used in this study was found to have 76 percent active site occupancy relative to total protein. The remaining active sites contained no metal ion. Substrates were purified prior to use by vacuum distillation. The highest purity grades of NADH and NAD^+ were purchased from either Sigma Chemical Company or Boehringer Mannheim Corporation and used without further purification. For kinetic isotope studies, both (4R)-4-deuterio-nicotinamide adenine dinucleotide (NADD) and isotopically normal NADH were prepared and purified as previously described [5, 8, 9]. Pyrazole (Aldrich) was purified by vacuum sublimation, isobutyramide (Aldrich) was purified by recrystallization from hot water. The rapid kinetics systems for rapid-scanning, rapid-mixing and for single-wavelength studies have been previously described [10, 11].

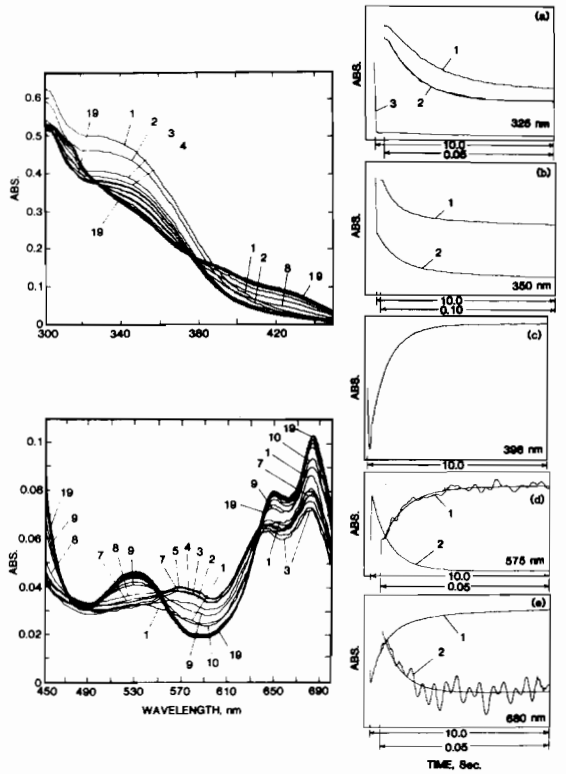

Fig. 1. Rapid-scanning, stopped-flow spectra for the Co(II)E-catalyzed reduction of benzaldehyde by NADH at 25 °C. Spectral acquisition times (measured in msec from the moment flow stopped): 1 (8.6), 2 (17.6), 3 (25.8), 4 (34.4), 5 (43.0), 6 (51.6), 7 (103.3), 8 (129.1), 9 (516.3), 10 (929.3), 11 (1979), 12 (4130), 13 (6282), 14 (9293), 15 (11875), 16 (14456), 17 (17038), 18 (19619), 19 (24782). (A) Conditions after mixing: (Syringe 1) [Co(II)E] = 34.4 μN; [EDTA] = 11.3 μM; [KCl] = 13.6 mM; (Syringe 2) [Benzaldehyde] = 0.955 mM; [NADH] = 47.4 μM; [pyr] = 24.3 mM; 0.1 M potassium glycinate buffer pH 9.04. (B) (Syringe 1) [Co(II)E] = 68.4 μN; [EDTA] = 10.4 μM; [KCl] = 23.9 mM; 25 mM potassium-TES buffer (pH 7 before mixing); (Syringe 2) [benzaldehyde] = 0.942 mM; [NADH] = 0.186 mM; [pyr] = 24.0 mM; 0.05 M potassium glycinate buffer, final pH 8.94. Insets (a)–(e) show single-wavelength time-courses. Conditions after mixing: (Syringe 1) [Co(II)E] = 53.6 μN; [EDTA] = 12.5 μM; [KCl] = 44.9 mM; (syringe 2) [benzaldehyde] = 0.951 mM; [NADH] = 0.107 mM; [pyr] = 24.2 mM; 0.1 M potassium glycinate buffer pH 9.04. Trace 2 in inset (a) was measured with 0.105 mM NADD in place of NADH.

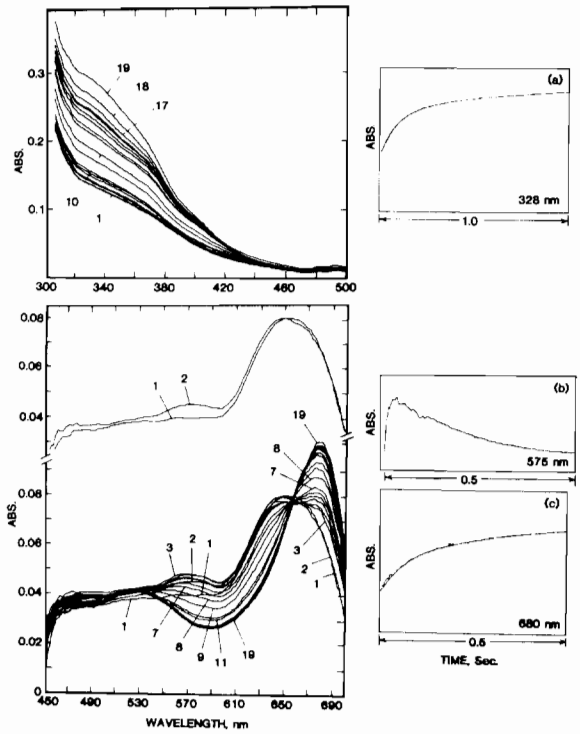

Fig. 2. Time-resolved spectra for the Co(II)E-catalyzed oxidation of benzyl alcohol by NAD^+ at 25 °C. Spectral acquisition times (msec): 1 (8.6), 2 (17.6), 3 (25.8), 4 (34.4), 5 (43.0), 6 (51.6), 7 (68.8), 8 (86.1), 9 (120.5), 10 (172.1), 11 (258.2), 12 (344.2), 13 (516.3), 14 (688.4), 15 (920.7), 16 (1119), 17 (1549), 18 (2409), 19 (4130). (A) Conditions after mixing: (Syringe 1) [Co(II)E] = 34.4 μN; [EDTA] = 11.3 μM; [KCl] = 13.6 mM; (Syringe 2) [benzyl alcohol] = 0.920 mM; [NAD^+] = 0.152 mM; [IBA] = 19.0 mM; 0.1 M potassium glycinate buffer pH 9.04. (B) (Syringe 1) [Co(II)E] = 95.7 μN; [EDTA] = 9.34 μM; [KCl] = 37.4 mM; (Syringe 2) [benzyl alcohol] = 2.10 mM; [NAD^+] = 1.58 mM; [IBA] = 43.5 mM; 0.1 M potassium glycinate buffer, pH 9.04. Insets (a)–(c) show single-wavelength time-courses. Conditions after mixing: [Co(II)E] = 66.7 μN; [KCl] = 31.0 mM; (syringe 2) [benzyl alcohol] = 2.10 mM; [NAD^+] = 1.48 mM; [IBA] = 43.5 mM; 0.1 M potassium glycinate buffer pH 9.04.

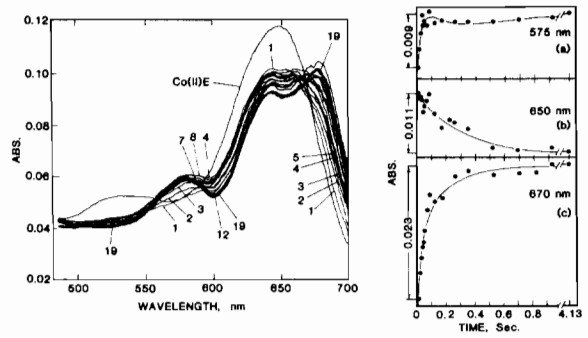

Fig. 3. Time-resolved spectra for the reaction of 1,1,1-trifluoroethanol (TFE) with the Co(II)E(NAD^+) complex at 25 °C. Concentrations after mixing: syringe 1, [Co(II)E] = 50.3 μN; syringe 2, [NAD^+] = 0.397 mM; [TFE] = 0.61 mM; [KCl] = 25 mM; 0.1 M potassium-glycinate buffer, pH 9.04. The rapid-scanning timing sequence is indicated in the insets. The scan rate was 8.605 msec/scan.

Results and Discussion

Reactions of horse liver alcohol dehydrogenase can be constrained to essentially a single turnover of sites when [coenzyme], [S] > [E] by the inclusion of fast-reacting, tight-binding inhibitors which bind selectively to either the E(NAD^+) or the E(NADH) complex. Pyrazole (pyr) has proven to be an extremely useful 'suicide' inhibitor for trapping the E(NAD^+) complex [3, 12, 13]. Isobutyramide (IBA) binds selectively to the E(NADH) complex and, at high concentrations, is effective in slowing the steady-state rate of alcohol turnover so that the first catalytic cycle is rapid relative to subsequent turnovers [14]. Using pyr and IBA, respectively, to trap the Co(II)E(NAD^+) and Co(II)E(NADH) complexes, we have investigated the single-turnover time-courses for aldehyde reduction and for alcohol oxidation.

Figures 1A and 1B show the families of time-resolved spectra during the reduction of benzaldehyde under single-turnover conditions obtained from RSSF experiments.The insets to these figures present single-wavelength time-courses at 325 nm (trace a), 350 nm (trace b), 398 nm (trace c), 575 nm (trace d) and 680 nm (trace e). These time courses establish that reaction occurs in two kinetically detectible relaxations. The spectral changes in the fast relaxation correspond to the oxidation of bound NADH (*viz.* Fig. 1A and traces a and b) and the appearance of a new d–d transition at 575 nm (Fig. 1B and trace d). Detailed analyses of the changes in Fig. 1A (not shown) indicate the fast process also is accompanied by perturbations of a LMCT band in the 350 nm region.

As is evidenced by the apparent isoabsorptive points at 325 nm and 380 nm, the slow relaxation consists of the conversion of the species formed in the fast phase to the Co(II)E(NAD-pyr) adduct (characterized by spectrum 19 in each of Figs. 1A and 1B). Consequently, we conclude that the species formed in the fast relaxation is a ternary complex containing NAD^+ and inner-sphere coordinated benzyl alcohol. Dissociation of benzyl alcohol from this complex is rate-limiting for the reaction of pyr with bound NAD^+.

Although there is a pronounced dependence of the fast and slow relaxation rates on substrate structure, the substrates investigated, acetaldehyde, benzaldehyde, anisaldehyde, cyclohexanone (this study) and *p*-nitrobenzaldehyde (Koerber *et al.*, in press) give spectral changes similar to those shown in Fig. 1. Substitution of NADD for NADH did not alter the

values of the measured rate constants for any of these substrates, compare traces 1 and 2 in inset (a) of Fig. 1. In contrast to these findings, the reduction of aromatic aldehydes catalyzed by the native Zn(II)E is characterized by a primary kinetic isotope effect when NADD is compared to NADH [12]. Consequently, these findings show that substitution of Co(II) for Zn(II) alters the rate limiting step for hydride transfer.

Figures 2A and B present RSSF data for the Co(II)E-catalyzed oxidation of benzyl alcohol. The first two spectra (collected respectively at 8.6 msec and 17.2 msec) are shown in Fig. 2B offset above the full set of spectra. Single wavelength time-courses at 328 nm (trace a), 575 nm (trace b) and 680 nm (trace c) are shown as insets. The spectra in Fig. 2A and the time-course in trace (a) show a burst appearance of NADH followed by a slow, steady-state appearance of more NADH as the IBA-inhibited enzyme recycles. The spectra in Fig. 2B and the time-course in trace (b) show that the burst appearance of NADH seen in Fig. 2A is *preceeded* by the appearance of a new d–d transition with $\lambda_{max} \cong 575$ nm. The decay of this 575 nm species to a steady-state in which the spectrum of the Co(II)E(NADH, IBA) complex dominantes, coincides with the burst appearance of NADH. These data indicate that a ternary complex containing NAD^+ and inner-sphere-coordinated benzyl alcohol is formed prior to hydride transfer. Similar spectral changes were obtained with ethanol, anisyl alcohol and cyclohexanol.

Attempts to model the 575 nm spectral changes found in these reactions led to the investigation of the reaction of an inhibitor, 2,2,2-trifluoroethanol (TFE), with the Co(II)E in the presence of NAD^+ (Fig. 3). We were surprised to find that the time-resolved spectra for the reaction of TFE with the Co(II)E-(NAD^+) complex occurs in two phases. During the fast phase, there appears a new 575 nm d–d transition. In the second, slower phase the new 575 nm band remains essentially unchanged while a new d–d transition appears at 680 nm.

The spectrum of the first Co(II)E(NAD^+, TFE) species (formed in the fast phase) appears to model the spectrum of the 575 nm intermediate formed prior to the oxidation of alcohol substrates (*viz.* Fig. 2), while the final (stable) Co(II)E(NAD^+, TFE) complex models the species formed upon reduction of aldehydes (*viz.* Fig. 1). The 575 nm d–d transition which characterizes all these Co(II)E(NAD^+, alc.) complexes also is present in the spectrum of the Co(II)E(NAD^+, acetate ion) complex, and in the spectrum of the Co(II)E(NAD^+) binary complex at pH 10.5.

From the above observations, we infer that the 575 nm band arises from inner-sphere coordination of oxy-anions to the active site cobaltus ion forming Co(II)E(NAD^+, RO^-) ternary complexes, where RO^- is the oxy-anion. If this interpretation is correct, then the transient 575 nm species detected in the single turnover reactions of aldehydes (Fig. 1) and of alcohols (Figs. 2 and 3) are Co(II)E(NAD^+, RO^-) ternary complexes. From similar reasoning, the spectrum of the Co(II)E(NAD^+) at pH 10.5 arises from coordination of hydroxide ion to the active site cobalt. Because no 575 nm band is present in the Co(II)-E(NAD^+) spectrum below pH 9, the apparent pK_a for the ionization of a coordinated water molecule must be $9.0 < pK_a < 10.5$.

Finally, we wish to draw attention to the apparent differences in the spectra of the Co(II)E(NAD^+, RO^-) species derived from aldehyde reduction (Fig. 1B) *vis à vis* the species formed during alcohol oxidation (Fig. 2B, spectrum 2). The transient formed in Fig. 1B is characterized by d–d transitions with $\lambda_{max} \cong$ 575 nm, 640 nm, and 680 nm, whereas the transient formed in Fig. 2B is characterized by d–d transitions with $\lambda_{max} \cong 575$ nm and 650 nm. Both of these species are formed when TFE reacts with the Co(II)-E(NAD^+) complex (Fig. 3); the species with $\lambda_{max} \cong$ 575 nm and 650 nm is formed as a transient, the species with $\lambda_{max} \cong 575$ nm, 640 nm and 689 nm is the stable product.

The Co(II)E d–d transitions in the 600–700 nm region are sensitive to the conformation state of the protein and/or to the coenzyme oxidation state [1, 2, 7]. Therefore, it is likely that the changes in this region of the spectrum which accompany the reaction of TFE signal changes in the Co(II) coordination sphere which result from conformational changes in the protein triggered by the coordination of TFE. Since it would appear that both types of alkoxide ion ternary complexes are kinetically competent [15], we propose that the protein conformation change for the interconversion of these complexes is an obligatory step in the catalytic mechanism of liver alcohol dehydrogenase.

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