High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill, p 221.

Pugmire, R. J., and Grant, D. M. (1968), J. Amer. Chem. Soc. 90, 4232.

Raftery, M. A., Huestis, W. H., and Millett, F. (1972), Cold Spring Harbor Symp. Quant. Biol. 36, 541.

Rajender, S., Lumry, R., and Han, M. (1971), J. Phys. Chem. 75, 1375.

Reynolds, W. F., Peat, I. R., Freedman, M. H., and Lyerla, J. R. (1973), J. Amer. Chem. Soc. 95, 328.

Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972), Biochemistry 11, 4293.

Robillard, G., and Shulman, R. G. (1972), J. Mol. Biol. 71, 507.

Smallcombe, S. H., Gammon, K. L., and Richards, J. H. (1972), J. Amer. Chem. Soc. 94, 4581.

Thompson, R. C., and Blout, E. R. (1973), Biochemistry 12, 44.

Wang, J. H. (1970), Science 161, 328.

Weigert, F. J., and Roberts, J. D. (1968), J. Amer. Chem. Soc. 90, 3543.

Whitaker, D. R. (1970), Methods Enzymol. 19, 599.

Whitaker, D. R., and Roy, C. (1967), Can. J. Biochem. 45, 911.

Wuthrich, K., Shulman, R. G., and Yamane, T. (1968), Proc. Nat. Acad. Sci. U. S. 61, 1199.

Estimate of Minimal Distance between Rapidly Exchanging Zinc and Nucleotide Binding Sites in Liver Alcohol Dehydrogenase[†]

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ABSTRACT: A hybrid horse liver alcohol dehydrogenase (Young, M., and Wang, J. (1971), J. Biol. Chem. 246, 2815) prepared by substituting cobalt(II) for the rapidly exchanging ("catalytic") zinc atoms shows an absorption maximum at 650 $m\mu$ and an enhanced absorption in the wavelength range from 300 to 450 $m\mu$. The appreciable spectral overlap between the absorption spectrum of this hybrid enzyme and the fluorescence emission of binary complexes with NADH, thionicotin-amide NADH, and Rose Bengal results in energy transfer from the bound ligand to the cobalt in the hybrid enzyme. Calculations based upon the Förster equation require a distance of at least 19 Å between the nicotinamide ring and the cobalt binding sites. These data make it unlikely that the readily exchanging zincs of liver alcohol dehydrogenase are directly involved in the catalytic process.

The role of zinc ions in the catalytic mechanism of horse liver alcohol dehydrogenase has been investigated by numerous workers (Plane and Theorell, 1961; Drum and Vallee, 1970a,b; Iweibo and Weiner, 1972). Weiner (1969) and Mildvan and Weiner (1969) have probed the active-site region of the dehydrogenase with a spin-labeled (nitroxyl) analog of ADP-ribose[1] which binds strongly to the NADH binding site. By studying the effect of the unpaired electron on the nitroxyl radical upon the proton resonance of ethanol, they were able to estimate the distance between the substrate alcohol and the ribosidic bond to the pyridine nitrogen of NADH. Proposed mechanisms for dehydrogenase activity have assumed that the zinc atoms at the active site are in close proximity to both nucleotide and ethanol; it has been postulated (Theorell and McKinley McKee, 1961) that the zinc atom binds portions of both substrate and cofactor as ligands. Recent data of Iweibo and Weiner (1972), however, have indicated that the strength of binding of NADH and NAD$^+$ is not changed when the dehydrogenase is stripped of all zinc. There are four zinc atoms per dehydrogenase molecule, two readily exchangeable and two only slowly replaceable (Drum et al., 1969). The loss of the rapidly exchanging zinc atoms is accompanied by a proportionate decrease in enzyme activity, and so it has been assumed that these "catalytic" zincs are situated near the active site. Iweibo and Weiner's (1972) finding that loss of both "catalytic" zinc and slowly exchanging structural zinc is not crucial for the binding of NADH suggests that even the "catalytic" zinc may also function to maintain appropriate structure at the active site.

Young and Wang (1971) have reported production of a hybrid alcohol dehydrogenase enzyme in which the two rapidly exchanging zinc ions are replaced by cobalt atoms. Use of this hybrid enzyme with cobalt in place of zinc together with an appropriate fluorescent donor (NADH or an NADH analog) held promise of estimating distances between the cobalt (or zinc) ions and the NADH moiety. Such distance calculations based upon use of the Förster equation have been extensively utilized by Latt et al. (1972) who studied cobalt quenching of fluorescence of synthetic carboxypeptidase substrates when cobalt is substituted for the native zinc in this metalloenzyme. It was hoped similarly that estimates of the distance between cobalt and the NADH molecule, in conjunction with the distance estimate of Mildvan and Weiner (1969), could be used to determine whether or not the cobalt

[1] Abbreviations used are: NAD⁻ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; $K_d K_i$, dissociation constant for the enzyme–inhibitor complex; sNADH, thionicotinamide analog of NADH; Hepes, N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid; ADP-ribose, adenosine diphosphate ribose; K_m, apparent Michaelis constant for substrate under discussion.

(or zinc) of dehydrogenase was close enough to either cofactor or substrate to perform its postulated role in the catalytic mechanism.

NADH and two appropriate fluorescent NADH analogs were chosen for this study. sNADH is a NADH analog with sulfur substituted for oxygen in the carboxamide of the nicotinamide. Anderson et al. (1959) synthesized sNADH and reported that it was an effective cofactor for the dehydrogenase. The other fluorescent analog chosen was the dye Rose Bengal. Brand et al. (1967) have reported that Rose Bengal is an inhibitor of the dehydrogenase reaction, competitive with respect to NADH. The dye shows the same stoichiometry of binding as NADH (two molecules per dehydrogenase molecule) and is readily displaced by NAD⁻ in the presence of pyrazole. These data indicate that the fluorescein derivative interacts with dehydrogenase at the NADH binding site. Rose Bengal is particularly well suited for study with cobalt-dehydrogenase as an energy acceptor since the fluorescence emission of the bound dye shows a high quantum yield and significantly overlaps the far-red absorption of the cobalt-dehydrogenase hybrid.

Materials and Methods

Chemicals. Horse liver alcohol dehydrogenase (crystalline suspension in 0.02 M phosphate buffer, pH 7.0) was purchased from Böehringer-Mannheim Corporation. Bovine serum albumin and horse liver alcohol dehydrogenase (1× crystallized and lyophilized), used solely as protein standards, were obtained from Worthington Biochemical Corporation. Nicotinamide adenine dinucleotide (grade III reduced and oxidized) was obtained from Sigma Chemical Co. Thionicotinamide adenine dinucleotide (oxidized) was obtained from PL Biochemicals, Inc. N-2-Hydroxyethylpiperazine-N-2'-ethanesulfonic acid (Hepes) buffer was obtained from Calbiochem. Phenol reagent (2 N) was obtained from Fisher Chemical Co. Isobutyramide was an Eastman Chemical Co. product.

Atomic absorption standards cobalt and zinc chloride (1000 ppm), quinine sulfate, and Rose Bengal were obtained from Matheson Coleman and Bell. Cobalt chloride and zinc acetate were reagent grade products of Mallinckrodt Chemical Works. Aquasol scintillation counting fluid and radioactive ⁶⁵Zn were obtained from New England Nuclear Corp. All other unspecified chemicals were reagent grade and were used without further purification.

Cobalt-Substituted Horse Liver Alcohol Dehydrogenase. Cobalt-containing hybrid dehydrogenase was prepared according to the procedures of Drum et al. (1969) and Young and Wang (1971). The dehydrogenase (60–75 μM), which was preequilibrated against 0.025 M Hepes (pH 7.0) buffer, was then dialyzed in cobalt containing buffers at pH 5.5 for varying lengths of time: buffer I consisted of 0.025 M sodium acetate and 0.1 M cobalt chloride at pH 5.5; buffer II consisted of 0.1 M sodium acetate and 0.2 M cobalt chloride at pH 5.5. After dialysis against the appropriate cobalt containing buffer in a nitrogen saturated atmosphere at 4°, the hybrid enzymes were dialyzed exhaustively against 0.025 M Hepes (pH 7.0). Care was taken to exclude air from the hybrid enzyme. Dehydrogenase protein determinations were made using the Lowry et al. procedure (1951).

Dehydrogenase with ⁶⁵Zn substituted for the rapidly exchanging "catalytic" zincs was prepared as described by Drum et al. (1969). The dehydrogenase (60–75 μM) was dialyzed 22 hr against 0.1 M sodium acetate (pH 5.5) plus 0.1 mM ⁶⁵ZnOAc (buffer III). The degree of ⁶⁵Zn incorporation

was monitored by scintillation counting in a Packard TriCarb liquid scintillation spectrometer (Model 3310). Small aliquots (0.1–0.2 ml) were counted in 10 ml of Aquasol.

Atomic Absorption. The metal content of the dehydrogenase was determined on a Varian-Techtron atomic absorption spectrophotometer (Model AA4). Standard curves for zinc and cobalt were prepared from 1000-ppm atomic absorption standards.

Enzymic Assay. Enzyme activity in the direction of NADH formation at pH 8.8 was followed on a Gilford Model 240 recording spectrophotometer. The assay system contained 1.67 mM NAD⁺, 16.6 mM sodium pyrophosphate (pH 9.0), and 17 mM ethanol. Enzyme activity in the direction of NAD⁻ formation was assayed at pH 7.0; the assay contained 0.1 M potassium phosphate at pH 7.0, 1.8 mM acetaldehyde, and 0.2 mM NADH. Specific activity was defined as nanomoles of substrate consumed per minute per milligram of protein at 25°.

Calculation of Energy Transfer. Calculations of the distances for transfer of electronic excitation energy from pyridine nucleotides or Rose Bengal to the cobalt of the hybrid alcohol dehydrogenase were made according to Förster (1965)

$$R_0{}^6 = 8.88 \times 10^{-25}(\kappa^2 \phi J/n^4) \tag{1}$$

where R_0 is the distance at which the probability of energy transfer equals the probability of spontaneous deactivation of the excited state, n is the refractive index, ϕ is the donor fluorescence quantum yield in the absence of energy transfer, κ^2 is the dipole–dipole orientation factor, and J is the overlap integral as shown in eq 2, where ϵ is the molar extinction co-

$$J = \frac{\int \epsilon(\nu)F(\nu)\mathrm{d}\nu/\nu^4}{\int F(\nu)\mathrm{d}\nu} \tag{2}$$

efficient of the acceptor, F is the fluorescence of the donor, and ν is the wave number. The efficiency of transfer between the donor and acceptor separated by a distance R was calculated from the following expression.

$$T = 1/[(R/R_0)^6 + 1] \tag{3}$$

The absorption spectra were taken with a modified Beckman DU spectrophotometer which incorporated a solid-state photometer and digital read-out system. Ten-centimeter cells were used in obtaining the spectra of the cobalt liver alcohol dehydrogenase.

Fluorescence measurements were made with a previously described spectrofluorimeter (Harvey et al., 1972) equipped with an Osram 450-W xenon arc lamp. Samples were maintained at 25° by means of a thermostated cuvet holder. Fluorescence studies with sNADH and Rose Bengal were performed with the image of the xenon lamp defocused from the entrance slit of the excitation monochromator; this attenuated light source minimized the possibilities of photodegradation of sNADH and photooxidation of the dehydrogenase by Rose Bengal. The optical densities of solutions were either less than 0.1 at the exciting wavelength or the fluorescence was appropriately corrected for the inner filter affect.

The data from binding and fluorescent experiments were fitted directly to appropriate theoretical curves using the reiterative procedure similar to that described by Wilkinson (1961).

The quantum yields of sNADH and of Rose Bengal bound

TABLE I: Time Course of Cobalt Incorporation into Liver Alcohol Dehydrogenase.[a]

Co Dialysis (hr)	Co/Dehydrogenase	Zn/Dehydrogenase	Σ_{metal}	Sp Act.
0		3.5	3.5	3.5
2	0.62	3.1	3.7	3.6
10	1.3	2.3	3.6	2.4
26	1.66	1.8	3.5	2.2
72	2.86	1.1	3.9	2.1
72[b]	3.0	0.5	3.5	1.7

[a] Results are expressed as moles of metal per mole of dehydrogenase (mol wt 80,000) as a function of time of dialysis in buffer I. [b] Dialysis in buffer II.

to zinc dehydrogenase were measured by comparison with standard solutions of quinine bisulfate in 0.1 N H_2SO_4 and riboflavine (pH 7), respectively. The quantum yields of quinine bisulfate and riboflavine were taken as 0.70 and 0.26, respectively (Scott et al., 1970; Weber and Teale, 1957).

Calibration of the xenon arc–excitation monochromator combination employed the method of Melhuish (1962) using a concentrated solution of Rhodamine B as a fluorescent screen. The detector system was calibrated by the method of Parker and Rees (1960) using a standard tungsten–iodide lamp.

Results

Characterization of the Hybrid Enzyme. Drum et al. (1969) have reported that only two "catalytic" zinc ions are exchanged in the dehydrogenase when metal exchange is conducted in buffer III. No further exchange of zinc occurs despite exhaustive dialysis under these conditions. Young and Wang (1971) have reported virtually complete substitution of cobalt for zinc when the dehydrogenase was dialyzed against 0.025 M sodium acetate containing 0.085 M cobalt chloride; two separate rates of exchange of cobalt for zinc were discerned. The time course of exchange of cobalt for zinc in buffer I is given in Table I. It can be seen that 2.9 of the 3.5 zinc atoms present in the native enzyme are replaced by cobalt after 72 hr of exchange under these conditions. During these experiments care was taken to ensure that the hybrid enzyme studied was "viable" according to two principal criteria: first, that the total number of metal binding sites was conserved; second, that the enzyme showed a specific activity >2. Enzyme which did not meet these criteria resulted primarily when the hybrid enzyme was exposed for extended periods to atmospheric oxygen.

The absorption spectrum of a hybrid dehydrogenase molecule containing 1.7 atoms of cobalt and 1.8 atoms of zinc per enzyme molecule is shown in Figure 1. The cobalt-containing enzyme, which is visibly green, exhibits a broad featureless absorption shoulder between 400 and 500 mμ with a distinct absorption peak at 650 mμ. The corresponding enzyme containing only zinc shows no absorption over this wavelength range.

Comparative kinetic studies were made on both native and hybrid dehydrogenases to determine whether significant changes in catalytic properties occurred upon cobalt substitution for zinc. The values of K_m for native enzyme and a
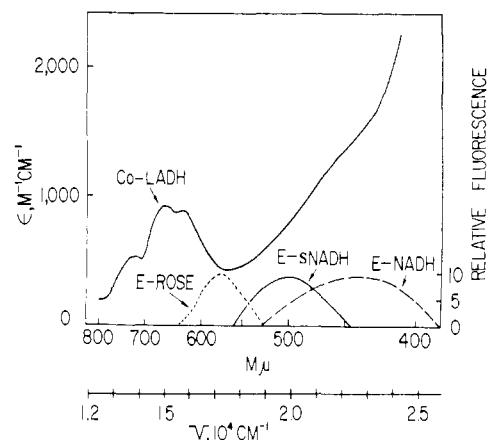

FIGURE 1: Spectral overlap of hybrid dehydrogenase and binary complexes with native dehydrogenase. Absorption spectrum of hybrid dehydrogenase (containing 1.7 and 1.8 atoms of cobalt and zinc, respectively, per enzyme molecule) in 0.025 M Hepes (pH 7.0) (upper curve). ε expressed in terms of molarity of enzyme molecules. Fluorescence emission spectra of 2 μM (---), 6 μM sNADH (—), or 0.5 μM Rose Bengal (···) in the presence of 12 μM native zinc–dehydrogenase in 0.025 M Hepes (pH 7.0). Excitation wavelengths were 340 (NADH), 400 (sNADH), and 500 mμ (Rose Bengal).

hybrid dehydrogenase containing 1.6 cobalt and 2.1 zinc atoms are shown in Table II. Also listed in this table are values for K_i for the inhibition of dehydrogenase by Rose Bengal. It can be seen from these data that the K_m and K_i values are similar for hybrid and native enzymes, indicating that no major differences in these enzymatic properties have resulted.

To ensure that the cobalt exchanged into enzyme was indeed going into "catalytic" zinc sites, [65]Zn was exchanged into the enzyme under conditions defined by Drum et al. (1969). This [65]Zn-labeled enzyme was used to determine whether stoichiometric removal of [65]Zn occurs simultaneously with cobalt incorporation into the protein. [65]Zn was exchanged for 26 hr in the buffer specified by Drum et al. (1969); this buffer contained 0.1 M sodium acetate (pH 5.5) and 0.1 mM [65]Zn acetate (buffer III). The enzyme was then dialyzed exhaustively against 0.025 M Hepes (pH 7.0) and checked for total zinc, radiolabeled zinc, and specific activity. This enzyme was subsequently dialyzed for 26 hr against cobalt-containing buffer I, dialyzed exhaustively against 0.025 M Hepes (pH 7.0), and then analyzed for cobalt, zinc and [65]Zn, and activity. The results shown in Table III indicate that 1.8 "catalytic" [65]Zn atoms exchanged into the dehydrogenase. Upon treatment with cobalt-containing buffer I, 1.7 cobalt atoms per dehydrogenase were incorporated into the enzyme. Simultaneously 1.6 "catalytic" [65]Zn atoms were lost from the enzyme. Thus, within experimental error, there was stoichiometric replace-

TABLE II: Kinetic Properties of Native and Hybrid Liver Alcohol Dehydrogenase.[a]

Enzyme	NAD+ → NADH		NADH → NAD+	
	K_m-(NAD+)	K_i(Rose Bengal)	K_m-(NADH)	K_i(Rose Bengal)
Cobalt hybrid	9	14.7	10	3
Native (zinc)	16.5	19	15.6	2.8

[a] Numbers indicate micromolar apparent Michaelis constants obtained at saturating concentrations of other substrates.

TABLE III: Comparison of Stoichiometry of Cobalt Substitution for "Catalytic" ^{65}Zn or Liver Alcohol Dehydrogenase.[a]

	Metal Content				
Enzyme Source	Zn/Dehy-drogenase	^{65}Zn/Dehy-drogenase	Co/Dehy-drogenase	Metal/Dehy-drogenase	Sp Act.
Native enzyme dialyzed against ^{65}Zn[a]	3.47	1.8		3.47	3.2
^{65}Zn enzyme (above) dialyzed against buffer I[b]	1.68	0.17	1.72	3.40	2.2

[a] Dialyzed 26 hr in buffer III and exhaustively against 0.25 M Hepes (pH 7.0). [b] Dialyzed 26 hr in buffer I and exhaustively against 0.25 M Hepes (pH 7.0).

ment of "catalytic" zincs by cobalt in 26 hr exchange in buffer I. When the degree of cobalt incorporation is limited to less than two cobalts per dehydrogenase, cobalt substitution occurs almost exclusively into the rapidly exchanging "catalytic" metal sites.

Binding of Rose Bengal to the Cobalt Liver Alcohol Dehydrogenase Hybrid. In order for there to be energy transfer between two chromophores, the emission of the donor in the absence of energy transfer must overlap the absorption of the acceptor. This spectral criterion is satisfied by the Rose Bengal and cobalt dehydrogenase hybrid couple (Figure 1); energy is transferred from the enzyme-bound dye to the cobalt substituted at the "catalytic" sites of the dehydrogenase. Such energy transfer would be seen experimentally as a decrease in the fluorescence of Rose Bengal bound to the hybrid when compared to the corresponding binary complex with the native zinc-containing enzyme.

Figure 2 shows that the expected quenching of Rose Bengal bound to the cobalt hybrid is experimentally realized. The data in Figure 2 correspond to a quenching of approximately 58%. The excitation maximum of Rose Bengal bound to the hybrid enzyme shows a 5–6-mμ red shift when compared to the corresponding maximum for the zinc-containing enzyme. This spectral shift could either result from minor differences in the structure of or the exact location of the Rose Bengal binding site; the significance of these differences is impossible to estimate. Rose Bengal bound to the zinc dehydrogenase shows a fluorescence enhancement of seven- to eightfold when
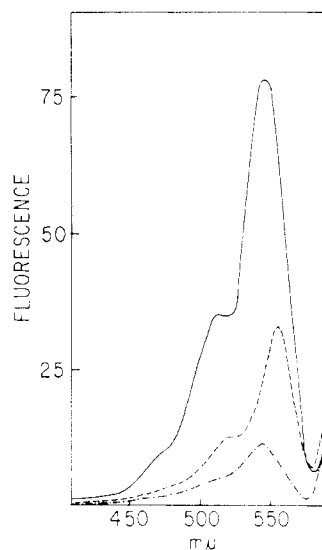
compared to the unbound dye; the possibility exists that the smaller fluorescence observed in the case of the hybrid enzyme is due to a lower affinity of Rose Bengal for the cobalt-substituted enzyme. This trivial possibility was excluded by experiments in which the relative fluorescence of Rose Bengal bound to zinc and hybrid enzymes was measured as a function of increasing protein concentrations. Dialysis against buffer I for 26 hr resulted in the incorporation of 1.7 atoms of cobalt per dehydrogenase molecule. When the reciprocal of fluorescence enhancement for the cobalt hybrid (Figure 3, line labeled 1.7 Co^{2+}) is extrapolated to infinite protein concentration, the ordinate intercept is different than that obtained with the zinc-containing enzyme. This difference indicates that when Rose Bengal is completely bound by the hybrid/enzyme (infinite number of binding sites relative to ligand concentration) its fluorescence is substantially less than that of the corresponding zinc enzyme. From the ordinate intercepts of Figure 3 it can be calculated that the 26-hr hybrid shows 66% quench relative to that of the corresponding zinc enzyme. It can be determined (Scatchard *et al.*, 1957) that the dissociation constant for the Rose Bengal–zinc dehydrogenase binary complex is 2.9 μM, while the corresponding dissociation constant for the cobalt hybrid binary complex is 2.4 μM. These dissociation constants are in good agreement with the kinetically

FIGURE 2: Fluorescence excitation spectra of 0.5 μM Rose Bengal: (----) free; (—) bound to native zinc–dehydrogenase (12 μM); (---) bound to hybrid enzyme (12 μM) described in Figure 1. Emission wavelength was 633 mμ.

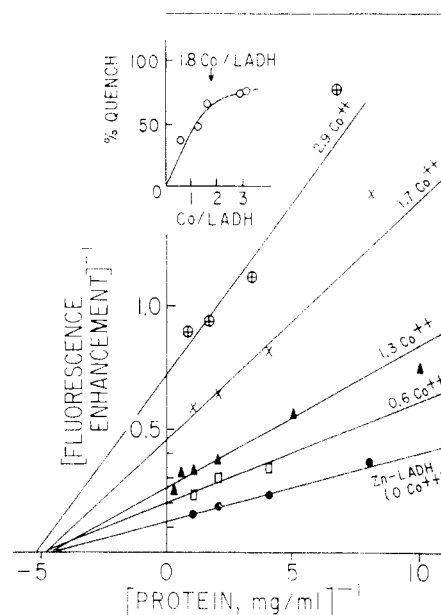FIGURE 3: Reciprocal of fluorescence enhancement of Rose Bengal (0.5 μM) *vs.* the reciprocal of dehydrogenase concentration as a function of degree of cobalt incorporation. Fluorescence enhancement is (fluorescence of Rose Bengal-dehydrogenase/fluorescence of unbound Rose Bengal) – 1. *i.e.*, an enhancement of 1.0 corresponds to a doubling of emission on interaction with enzyme.

TABLE IV: Summary of Fluorescence Quenching Data.

Enzyme Ligands	Quantum Yield	$10^{-16}J$ (cm^6/ mmol)	R_0 (Å)	% Quench	R (Å)
Rose Bengal	0.17	38	21	66	19
NADH	0.057	30	17	33	19
sNADH	0.0014	27	9	0	17
NADH–isobuty-ramide	0.17	30^a	20	32	23
Rose Bengal-acetaldehyde	0.11	38^a	19	30	23

^{a} Overlap integral assumed to be same as that of corresponding binary complex.

determined K_i (Table II) for the zinc- and cobalt-containing hybrid as well as with the K_d determined by Brand *et al.* (1967) for native zinc enzyme.

Figure 3 shows the quenching of Rose Bengal bound to a series of dehydrogenase hybrids differing in the extent of substitution of cobalt for the zinc of the native enzyme. The degree of quench calculated from the reciprocal of fluorescence enhancement extrapolated to infinite protein is shown in the inset to Figure 3. The extent of energy transfer (quenching) from Rose Bengal to the cobalt of the hybrid enzyme increases as the 1.8 atoms rapidly exchanging zinc are replaced by cobalt. Further substitution of cobalt for zinc atoms of dehydrogenase (replacement of structural zincs) results in little additional fluorescence quenching of bound Rose Bengal (from 66% quench at 1.8 Co per dehydrogenase to 75% quench at 3.0 Co per dehydrogenase). This suggests that the latter, slowly exchanging "structural" zinc atoms are more than 35 Å from the Rose Bengal (NADH) binding site, *i.e.*, too far away from bound dye to produce significant quenching.

Calculation of the distance between Rose Bengal and the cobalt of the hybrid dehydrogenase enzyme (containing 1.7 cobalt atoms per dehydrogenase molecule) was performed using eq 1–3 and the data of Table IV ($\kappa^2 = {}^2/_3$ and the index of refraction taken to be 1.5 (Latt *et al.*, 1970)). The calculated distance between the enzyme-bound Rose Bengal and the cobalt atom of the hybrid dehydrogenase molecule is 19 Å. However, ambiguity exists as to the exact location of the emission oscillator of the Rose Bengal molecule. Also unknown is the correspondence of the binding site for Rose Bengal and the binding site for the nicotinamide portion of NADH. Thus, it seemed advisable to confirm this distance estimate with fluorescent nucleotides which are substrates for the dehydrogenase reaction.

Binding of NADH and sNADH to the Cobalt Hybrid Enzyme. It can be seen from Figure 1 that both NADH and the thionicotinamide analog of NADH bound to the dehydrogenase have emission spectra which overlap with the absorption spectrum of the cobalt dehydrogenase hybrid. Thus, the spectral properties are compatible with energy transfer from the pyridine nucleotide to the cobalt of the hybrid dehydrogenase. The fluorescence emission of NADH bound to the cobalt dehydrogenase (extrapolated to infinite protein concentration) is quenched 33% when compared to the limiting fluorescence observed with the zinc enzyme (Figure 4). In contrast, when the fluorescence emission of the thionicotinamide analog is extrapolated to infinite protein
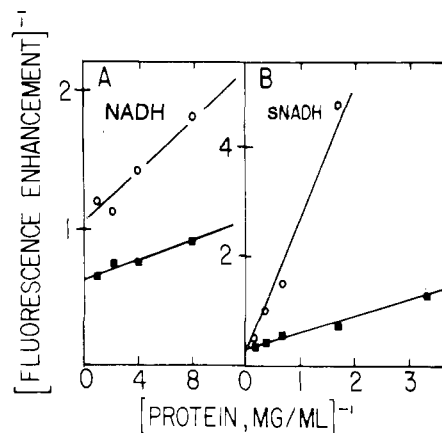

FIGURE 4: Reciprocal of fluorescence enhancement *vs.* reciprocal of dehydrogenase concentration for NADH (2 μM) and sNADH (6 μM): (○) cobalt hybrid containing 1.7 atom of cobalt per dehydrogenase; (□) native zinc–dehydrogenase.

concentration there is no difference in the limiting fluorescence for the cobalt- and zinc-containing dehydrogenase enzymes. Calculation of the distance between NADH and the cobalt of the hybrid dehydrogenase using the data of Table IV yields a distance estimate of 19 Å. The thionicotinamide data indicate only that the minimal distance between metal and nicotinamide ring is greater than 17 Å. The NADH-derived estimate is in good agreement with the distance calculated with Rose Bengal; the absence of quenching observed for thionicotinamide is consistent with this distance estimate of 19 Å.

While the above data indicate a distance of 19 Å between the rapidly exchanging metal in the dehydrogenase and either Rose Bengal or NADH, ternary complex formation occurring during catalysis may alter the conformation of the active site (Zeppezauer *et al.*, 1967). The fluorescence quenching of NADH during substrate turnover has not been measured; however, fluorescence quenching of ternary complexes formed with the inhibitor isobutyramide and NADH has been investigated. This ternary complex (Table IV) shows 32% quenching of nucleotide fluorescence when hybrid enzyme is compared to native enzyme. This quenching corresponds to a distance of 23 Å between the nicotinamide moiety of NADH and the cobalt atom of the ternary enzyme complex.

The addition of acetaldehyde to the Rose Bengal-dehydrogenase ternary complex results in a ternary complex which is analogous to the isobutyramide–NADH complex described above. On extrapolation to infinite concentration of acetaldehyde the fluorescence of Rose Bengal in the cobalt-containing ternary complex is quenched 30% relative to the corresponding complex formed with the Zn–dehydrogenase; this quenching indicates a distance of 23 Å between bound Rose Bengal and the cobalt of the hybrid enzyme, a value which is in good agreement with the data obtained with the NADH–isobutyramide ternary complex.

Discussion

The distinction between "catalytic" zinc atoms and "structural" zinc atoms is based upon differences in the rate of exchange of the metal ions at pH 5.5. This study was focused upon the "catalytic" (rapidly exchanging) zincs because of their proposed role as general acids in the enzymic oxidation of ethanol (Abeles *et al.*, 1957). Care was taken to replace only "rapidly exchanging" zinc atoms with cobalt in order to
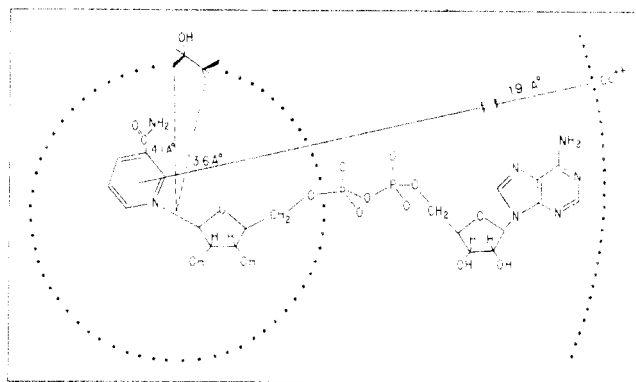
FIGURE 5: Schematic representation of distances between enzyme-bound substrate, nucleotide, and metal.

eliminate the possibility of multiple interactions between NADH (or the NADH analog) and cobalt substituted at *both* "catalytic" and "structural" sites.

The conditions of metal exchange employed result in a hybrid enzyme containing equivalent molar amounts of cobalt and zinc. Considering the estimated error in the metal determination, these experiments would indicate that at least 90% of the cobalt was incorporated at the two metal binding sites defined operationally as rapidly exchanging, *i.e.*, those sites which exchange with ^{65}Zn at pH 5.5 (Table III). It is conceivable that the remaining 10% (or less) of the cobalt was bound at the slowly exchanging metal binding sites of the dehydrogenase. The quenching of ligand fluorescence observed on binding to the hybrid would then be due to energy transfer from ligand to cobalt substituted at both the rapidly and slowly exchanging sites. However, energy transfer to the small amount of cobalt postulated to be bound at the slowly exchanging site would not significantly alter the distance calculations. For example, suppose the 66% quenching observed with Rose Bengal bound to the hybrid enzyme containing 1.7 Co and 1.8 Zn atoms per dehydrogenase resulted from partial quenching by the rapidly exchanging cobalt and complete quenching by the small amount ($\leq 10\%$ of total cobalt) of slowly exchanging cobalt. In this case, the true quenching of Rose Bengal fluorescence due to interaction with rapidly exchanging cobalt would be 62% which is experimentally indistinguishable from the observed value of 66% quenching. This altered value of 62% is calculated assuming that the observed quenching of fluorescence can be represented as a weighted summation of quenching by cobalt at the rapidly exchanging and slowly exchanging metal binding sites. Conversely, if the slowly exchanging cobalt *does not quench*, the corrected quenching by the rapidly exchanging cobalt will be 74%, which corresponds to a distance of approximately 17 A. Thus, the presence of a hypothetical 10% contamination by slowly exchanging cobalt can potentially introduce an error of only ~2 A in the distance between the rapidly exchanging cobalt and Rose Bengal. Similar arguments hold for distance calculations using NADH and sNADH. Thus, provided one accepts Vallee's operational definition of slowly and rapidly exchanging zinc as a meaningful criterion for differentiating two distinct classes of metal binding sites in dehydrogenase, the estimated distance between metal and NADH or Rose Bengal is subject to a $=10\%$ uncertainty as a result of possible population of slowly exchanging metal sites by cobalt. Even the quenching of fluorescence observed for NADH or the NADH analog by

cobalt substituted solely at the two fast exchanging metal sites may derive from multiple interactions between cobalt ions and fluorescent cofactors. Depending upon the distances between metal binding sites and cofactor binding sites, one may observe quantitatively greater quenching of fluorescence than expected for exclusively pairwise interactions between the NADH analog and cobalt. Thus, the distance estimates based upon the Förster equation represent minimal estimates of the distance between interacting species.

Nonradiative energy transfer from NADH (or the NADH analog) to cobalt dehydrogenase is observed as a reduction in the fluorescence enhancement of ligand bound to hybrid enzyme relative to that observed upon binding of the coenzyme to the zinc enzyme. Since the cobalt of hybrid dehydrogenase is not fluorescent, no sensitized fluorescent emission can be demonstrated. The quenching of fluorescence by the hybrid enzyme relative to the wholly zinc-containing enzyme could be due to factors other than energy transfer. Thus it seemed desirable to obtain more than one estimate of the distance between metal ion and cofactor in order to show that nonspecific quenching effects were probably not a significant factor in the relative fluorescence quenching observed. The data of Table IV indicate a very good agreement between distance estimates obtained with NADH and Rose Bengal, both of which are consistent with the data obtained with sNADH. The agreement of the data for Rose Bengal and NADH is undoubtedly, in part, fortuitous for several reasons. First, it is impossible to specify *a priori* the position of the Rose Bengal emission oscillator with respect to the actual site occupied by the nicotinamide emission oscillator. Second, a value of $\frac{2}{3}$ has been assumed for κ^2 in calculating distances utilizing the Förster equation. This assumption is probably reasonable for the case of Rose Bengal since the fluorescence emission of the dye predominantly overlaps the long-wavelength (beyond 550 mμ) absorption arising from the degenerate d–d transition of the Co–dehydrogenase complex. However, the fluorescence emission of NADH overlaps the Co dehydrogenase absorption in the 350–500-mμ wavelength region which probably represent charge transfer bands. Because charge transfer transitions can show highly directional characteristics, in this case κ^2 can assume its full range of values between 0 and 4. If the true value of κ^2 were 4 instead of $\frac{2}{3}$, this would only result in a 30% underestimate in R, due to the sixth root dependence of R upon κ^2. If, however, the true κ^2 were very small, approaching zero, the probability of energy transfer would approach zero resulting in a large overestimate of R. For example, $\kappa^2 = 0.01$ would correspond to $R_0 = 9$ A instead of 19 A. Charge transfer bands, being potentially more anisotropic than the d–d transitions in the visible spectrum of Co^{2+}, could conceivably show such an unfavorable orientation of emission and acceptor oscillators. It is impossible, of course, to know *a priori* whether or not such a highly unfavorable orientation of donor acceptor oscillators does in fact occur in the case of NADH complexes of cobalt–dehydrogenase. However, the agreement of distance estimates utilizing both NADH and Rose Bengal leads us to conclude that such an unfavorable situation does not, in fact, obtain for the quenching of NADH fluorescence by cobalt–dehydrogenase.

The contamination of the cobaltous enzyme by some cobaltic (Co(III)) dehydrogenase complex (which absorbs strongly in the 350–500-mμ range) could also result in an erroneously large value for R_0 due to an increase in the overlap integral, J. However, this consideration is probably not quantitatively important both because of the sixth-root dependence

of R_0 on J and because the hybrid was prepared under anaerobic conditions. However, it is not possible to unambiguously assign an estimate of probable error due to partial oxidation of cobaltous to cobaltic at this time.

The distance between the nicotinamide ring of NADH and cobalt ion is estimated to be at least 19 Å. Mildvan and Weiner (1969) have estimated the distance between the methyl and methylene protons of ethanol and the ribosidic bond of the pyridine nitrogen to be 3.6 and 4.1 Å, respectively. The relationships between the geometry of the dehydrogenase active site as delineated by Mildvan and Weiner (1969) relative to the distances demonstrated in this work are shown in Figure 5.

Even under the most favorable conditions when ethanol lies between cobalt and Rose Bengal the distance between the ethanol and cobalt is too far for the meaningful interactions of the "catalytic" cobalt and ethanol to occur. The "catalytic" cobalt thus appears to be too far away to directly participate in catalysis. Based on the above considerations it would appear that the faster exchanging zinc ions play a structural and not a catalytic role in the dehydrogenase.

However, Zeppezauer et al. (1967) have shown that, in addition to conformational changes occurring upon binding of NADH to the dehydrogenase, a subsequent conformational change occurs upon formation of the ternary complex between dehydrogenase–NADH and isobutyramide. Thus, it was possible that upon formation of the ternary complex, a further alteration of enzyme structure might occur which brought the ethanol or acetaldehyde in closer proximity to the cobalt. To test this hypothesis, fluorescent quenching in ternary complexes was investigated. The distance estimate based upon the fluorescence quenching in ternary complexes is shown in Table IV. It can be seen that the distance between metal and cofactor actually increases in the ternary complex.

Thus, utilizing the active-site distance estimates of Mildvan and Weiner (1969), together with our own estimates of distance between "catalytic" zinc atoms and NADH, it is concluded that the distinction between "catalytic" and "structural" zincs may not be valid. The results of this study would indicate that rapidly exchanging zincs are no more "catalytic" than are the slower exchanging zincs. This result is in agreement with the findings of Young and Wang (1971) which were based upon substrate and cofactor binding by cobalt hybrid and apoenzyme, respectively. However, recent X-ray diffraction studies done at 2.9-Å resolution (C. I. Bränden, personal communication) have resulted in location of the four zinc atoms relative to the position of the active sites. These data indicate that the "structural" and "catalytic" zincs are approximately 20 Å apart, with the "catalytic" zinc lying in the active-site cleft. Studies using the ADP-ribose–dehydrogenase binary complex indicate that the nicotinamide ring lies quite close to the active-site ("catalytic") zinc. The reason for this apparent disagreement between the X-ray diffraction data and results of this study is not readily apparent. The X-ray data obtained thus far have been done on crystals of the isolated enzyme or upon binary complexes of the enzyme which crystallize in the same space group. It is known that binding of NADH causes a conformational change which alters the crystal symmetry (Branden et al., 1969). Thus the disparity of conclusions could stem from conformational differences caused by binding of NADH or by differences between crystal and solution conformations of the enzyme.

Theorell and McKinley McKee (1961) have proposed that the zinc ion coordinates, as a ligand, the free amino group of adenine. If the NADH molecule is in the folded configuration then our data would exclude this possibility with respect to the rapidly exchanging zincs. If, on the other hand, the NADH molecule is in an extended configuration then it would be possible that the adenine amino group could interact with the zinc ion. However, Iweibo and Weiner (1972) have shown that ADP-ribose and a nitroxyl spin-label analog of ADP-ribose show equal affinity for both native and apoenzyme. This is strong evidence that the zinc atom is in fact not an integral part of the binding site for the adenine moiety of NADH; their data, in concert with ours, would indicate that neither the nicotinamide nor the adenine portions of NADH interact with the rapidly exchanging zinc atoms in dehydrogenase.

References

Abeles, R. H., Hutton, R. F., and Westheimer, F. (1957), J. Amer. Chem. Soc. 79, 712.

Anderson, B. M., Ciotti, C. J., and Kaplan, N. O. (1959), J. Biol. Chem. 234, 1219.

Brand, L., Gohkle, J. R., and Rao, D. S. (1967), Biochemistry 6, 3510.

Branden, C. I., Zepperzauer, E., Boiwe, T., Soderlund, G., Soderberg, B. O., and Nordstrom, B. (1969), in Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Springer-Verlag, p 129.

Drum, D. E., Li, T. K., and Vallee, B. L. (1969), Biochemistry 8, 3792.

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

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

Förster, T. (1965), in Modern Quantum Chemistry, Part III, Sinanoglu, O., Ed., New York, N. Y., Academic Press, p 93.

Harvey, R. A., Heron, J. I., and Plaut, G. W. E. (1972), J. Biol. Chem. 247, 1801.

Iweibo, I., and Weiner, H. (1972), Biochemistry 11, 1003.

Latt, S. W., Auld, D. S., and Vallee, B. L. (1970), Proc. Nat. Acad. Sci. U. S. 67, 1383.

Latt, S. W., Auld, D. S., and Vallee, B. L. (1972), Biochemistry 11, 3015.

Lowry, D. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), J. Biol. Chem. 193, 265.

Melhuish, W. H. (1962), J. Opt. Soc. Amer. 52, 1256.

Mildvan, A. S., and Weiner, H. (1969), J. Biol. Chem. 244, 2465.

Parker, C. A., and Rees, W. T. (1960), Analyst 85, 587.

Plane, R. A., and Theorell, H. (1961), Acta Chem. Scand. 15, 1866.

Scatchard, G., Coleman, J. S., and Shen, A. L. (1957), J. Amer. Chem. Soc. 79, 12.

Scott, G. T., Spencer, R. D., Leonard, N. J., and Weber G. (1970), J. Amer. Chem. Soc. 92, 687.

Theorell, H., and McKinley McKee, J. S. (1961), Acta Chem. Scand. 15, 1834.

Weber, G., and Teale, F. W. J. (1957), Trans. Faraday Soc. 53, 646.

Weiner, H. (1969), *Biochemistry 8*, 526.

Wilkinson, G. N. (1961), *Biochem. J. 30*, 324.

Young, J. M., and Wang, J. H. (1971), *J. Biol. Chem. 246*,

2815.

Zeppezauer, E., Soderberg, B. O., Branden, C. I., Akeson, A., and Theorell, H. (1967), *Acta Chem. Scand. 21*, 1099.

A Calorimetric Study of the Interactions between Phosphorylase *b* and Its Nucleotide Activators†

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ABSTRACT: Interactions of glycogen phosphorylase *b* with AMP and IMP have been studied by calorimetry at two different temperatures: 18 and 25°. Calorimetric titration of the enzyme with AMP shows a biphasic curve suggesting that the enzyme contains two sets of AMP binding sites with different affinity. The high affinity sites are essentially filled at 0.3 mM AMP whereas significant nucleotide binding at the low affinity sites occurs only when AMP concentration exceeds 1 mM. Since phosphorylase *b* is maximally activated at 1 mM AMP, the low affinity sites are probably not directly involved in the AMP activation of the enzyme. The heat of interaction between phosphorylase *b* and 0.5 mM AMP has been measured calorimetrically as a function of the enzyme concentration. This heat per mole of the enzyme increases as the enzyme concentration increases at 18° but not at 25°. Sedimentation velocity experiments have shown that phosphorylase *b*, in the presence of 0.5 mM AMP, undergoes reversible changes between a dimeric and a tetrameric species. These results suggest that the heat of phosphorylase *b*–AMP interaction at 18° consists of the heat of AMP binding and that of the

tetramer formation. The enthalpies of the AMP binding and the tetramer formation, as well as the equilibrium constant for the enzyme association calculated from the calorimetric data at 18°, are respectively 2.6 kcal/mol, −85 kcal/mol, and 2.1×10^5 M^{-1}. At 25°, the enthalpy of AMP binding to phosphorylase *b* is found to be −13.2 kcal/mol. In contrast to AMP, IMP does not induce phosphorylase *b* association either at 25 or at 18°. The enthalpy of IMP binding to phosphorylase *b* is about −11 kcal/mol, independent of the temperature. Changes in free energy, enthalpy, and entropy accompanying the AMP induced phosphorylase *b* association at 18° have been calculated from the calorimetric data to be respectively −7.1 kcal/mol, −85 kcal/mol, and 268 eu. These thermodynamic properties indicate that the driving force for the AMP induced enzyme association is enthalpic. Thermodynamic parameters for bindings of AMP and IMP to phosphorylase *b*, both at 18 and 25° have also been calculated. The results indicate that conformational changes in phosphorylase *b* induced by AMP at 18° are different from those induced by IMP and those induced by AMP at 25°.

S keletal muscle glycogen phosphorylase *b* depends on AMP for catalytic activity (Green and Cori, 1943). Both V_{max} and K_m of the enzyme are functions of the nucleotide concentration. The interactions between the nucleotide activator and phosphorylase *b* have been studied under a variety of conditions by kinetic and equilibrium binding techniques (Madsen and Shechosky, 1967; Buc and Buc, 1967; Kastenschmidt *et al.*, 1968a,b). Using conformational probes, it has been shown that the binding of AMP to phosphorylase *b* is accompanied with conformational changes (Ullman *et al.*, 1964; Buc and Buc, 1967; Birkett *et al.*, 1971), as well as with an enhanced tendency of the enzyme to associate into a tetramer (Appleman, 1962; Sealock and Graves, 1967; Wang *et al.*, 1968). In addition to AMP, IMP and several other nucleotides also activate phosphorylase *b* (Cori *et al.*, 1938; Mott and Bieber, 1968; Okazaki *et al.*, 1968). These activators differ from AMP in that they do not significantly affect the K_m of phosphorylase *b* toward glucose 1-phosphate, nor do they cause the enzyme to associate into a tetramer unless the substrate glucose 1-phosphate or phosphate is also present (Black and Wang, 1968; 1970; Mott and Bieber,

1970). These observations have led to the suggestion that conformational changes of phosphorylase *b* induced by AMP differ from those induced by IMP or other nucleotides (Black and Wang, 1968, 1970).

In spite of extensive studies on nucleotide activation of phosphorylase *b*, information concerning the thermodynamics of the interactions are meager. Kastenschmidt *et al.* (1968a,b) have calculated some thermodynamic parameters for AMP binding to phosphorylase *b* based on kinetic and equilibrium binding data. In our laboratory, microcalorimetric measurements of the heat of interactions between phosphorylase *b* and AMP have been initiated and some preliminary results have been reported (Wang *et al.*, 1970). The present communication concerns itself mainly with the thermochemistry of phosphorylase *b*–AMP interaction and that of the AMP induced enzyme association. The results on calorimetric study of phosphorylase *b*–IMP interaction are also included.

Materials and Methods

Glycogen phosphorylase *b* was prepared according to the procedure of Fischer and Krebs (1962), and was recrystallized four times in 0.04 M glycerophosphate–0.03 M cysteine buffer (pH 6.8) containing 1 mM AMP and 10 mM Mg(OAc)₂. For calorimetric and ultracentrifugal studies, phosphorylase *b* preparations were used within 1 week after the fourth crystal-