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Short Communications

Fluorometric Observations of Binary and Ternary Complexes Formed with Malic Dehydrogenase, Coenzyme, and D-Malic Acid

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Following the observation by Boyer and Theorell [1] of a change in the fluorescence spectrum of DPNH ** on binding to horse liver alcohol dehydrogenase, enzyme-reduced pyridine nucleotide complexes have been detected fluorometrically with yeast alcohol dehydrogenase[2], lactic[3], glutamic[4], and *iso*citric dehydrogenase [5]. With several of these enzymes fluorometric changes also occur on formation of ternary complexes of enzyme, reduced coenzyme, and substrate analogues. These phemomena have now been observed with malic dehydrogenase by Pfleiderer *et al.*[6,7] and independently in this laboratory. This communication is a report of the changes which occur in the fluorescence spectrum of DPNH on binding to malic dehydrogenase, and on formation of ternary complexes of malic dehydrogenase, DPNH, and D-malic acid. The binding of coenzyme and malic acid in binary and ternary complexes has been studied quantitatively by means of these changes.

Experimental. The fluorometric observations were carried out in the spectrofluorometer previously described [8], and calculations were made using equations based on those employed earlier in this laboratory [8,9]. All measurements were made at 23.5°C in 0.1 μ phosphate buffer containing 10^{-3} M EDTA. The pH was 7.15 unless otherwise noted. Titrations were followed using a measuring wavelength of 420 mμ and an activation wavelength of 352 mμ. Emission and excitation maxima are uncorrected for variations with wavelength in the intensity of the exciting light and sensitivity of the photocell. The correction would move the maxima about 15 mμ towards shorter wavelengths on this apparatus. Pig heart malic dehydrogenase was the product of C. F. Boehringer and Soehne, G.m.b.H. * and was assayed according to Ochoa [10]. DPN and DPNH were obtained from Sigma Chemical Co. and D- and L-malic acid from the California Corp. for Biochemical Research.

Results and discussion. Fig. 1 shows the changes in the fluorescence spectrum of DPNH on binding to malic dehydrogenase, and the further change in the spectrum on the addition of D-malic acid. In the presence of enzyme the observed emission maximum of DPNH shifts from 478 mμ to 470 mμ and doubles in intensity. On addition of 50 mM D-malate the maximum shifts further to 440 mμ and is increased an additional three fold. Changes in the fluorescence of enzyme-bound DPNH produced by substrate analogues or reduced substrates were originally observed with

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** Abbreviations: DPN and DPNH, oxidized and reduced forms, respectively, of diphosphopyridine nucleotide; EDTA, ethylenediamine tetra-acetic acid. The constants, K, are the dissociation constants of the species whose abbreviations are separated by commas in the subscript. Subscript abbreviations: E, enzyme; R, DPNH; O, DPN; I, D-malate; ER, EO, and EI, binary complexes of enzyme with DPNH, DPN, or D-malate; *e. g.* $K_{EI,R}$ is the dissociation constant for the reaction $EI + R \rightleftharpoons EIR$.

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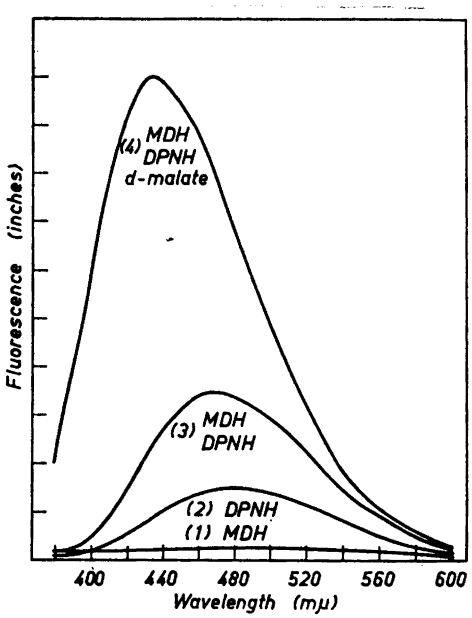

Fig. 1. Fluorescence emission spectrum of DPNH-malic dehydrogenase complex, and the ternary complex of malic dehydrogenase, DPNH, and D-malic acid. Curve (1): 3.6×10^{-4} units of malic dehydrogenase. Curve (2): 3.3×10^{-6} M DPNH. Curve (3): 3.3×10^{-6} M DPNH plus 3.6×10^{-4} units of malic dehydrogenase. Curve (4): 3.3×10^{-6} M DPNH, 3.6×10^{-4} units of malic dehydrogenase, and 5×10^{-2} M D-malate. Activating wavelength 352 mμ. Buffer, 0.1 μ phosphate, pH 7.15, containing 10^{-3} M EDTA. Final volume, 2 ml. Temp., 23.5°C.

lactic dehydrogenase [3], and have since been detected with a number of other dehydrogenases [3,4,11,12]. Kinetic and equilibrium data [9,12] have supported the view that the fluorescence changes result from the formation of ternary complexes of enzyme, coenzyme and substrate analogue.

Addition of L-malate to the malic dehydrogenase-DPNH complex produced no change in fluorescence. However, L-malate competitively displaced D-malate from the ternary complex, producing a return to the original DPNH-enzyme spectrum when added in large excess. Small changes in the fluorescence of the enzyme-DPNH complex were produced by succinate and malonate.

The activation spectra for the fluorescence of the DPNH and D-malate complexes are shown in Fig. 2. The excitation maximum for DPNH fluorescence is seen to increase from 352 mμ to 362 mμ on combination with the enzyme. This is in contrast to the changes which have been observed with other complexes, in which the activation maximum decreases [4,5,14] or is unchanged [12]. On the addition of D-malic acid, there is a decrease in the excitation maximum to 345 mμ, a value below that of free DPNH.

Previous studies on horse liver alcohol dehydrogenase by Theorell and Winer [8,9] have utilized fluorescence changes for the quantitative study of coenzyme binding and ternary complex formation. This technique now has been applied to malic dehydrogenase. The enzyme was found to bind 4.4 moles of DPNH per mole, based on the turnover number of 38 000 moles of DPNH per mole enzyme per min reported by Wolfe and Neilands [15] for the conditions of the assay used in this work *. The binding of DPNH and DPN was competitive. No significant deviations from simple monovalent dissociation were observed, indicating that the binding sites act independently and equally. At pH 7.15, $K_{E,R}$ was 1.0 μM and $K_{E,O}$ 280 μM. From titrations in the presence of D-malate, $K_{EI,R}$ was found to be 0.4 μM, $K_{ER,I}$ 6 000 μM and $K_{E,I}$ 15 000 μM. At high concentrations of D-malate, 80 to 160 mM, $K_{EI,R}$ increased 30 to 50 %, apparently due to the effect of ionic strength. The mutual stabilizing effect of DPNH and D-malate on the binding of one another, shown by the fact that $K_{EI,R} < K_{E,R}$ and $K_{ER,I} < K_{E,I}$, indicates that a ternary complex, in which the bound molecules interact, is formed. D-Malate interacted specifically with DPNH. DPN and D-malate were bound to the enzyme without interaction, since the values found for $K_{EI,O}$, 299 μM, and $K_{EO,I}$, 14 900 μM, are in close agreement with $K_{E,O}$ and $K_{E,I}$. L-Malate apparently has the opposite specificity. It was without effect on the dissociation of DPNH, although it presumably interacts in the binding of DPN in the enzymatically active complex.

Preliminary measurements have been carried out at pH 6.0 and 8.0. The changes in the dissociation constants of the binary

* Pfleiderer has reported [6,7] that his malic dehydrogenase preparations bind one mole of DPNH per 40 000 g of protein, the molecular weight according to Neilands [15].

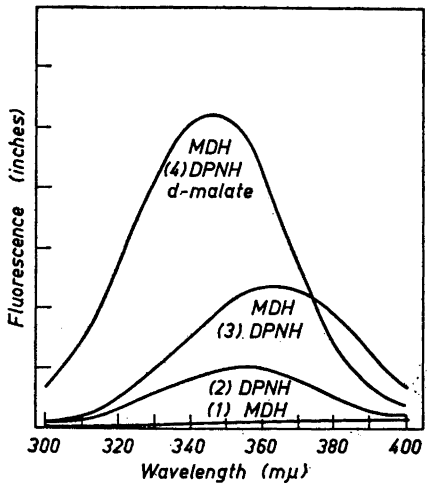

Fig. 2. Activation spectrum for fluorescence of DPNH, DPNH-malic dehydrogenase complex, and the ternary complex of malic dehydrogenase, DPNH, and D-malic acid. Curve (1): 3.6×10^4 units of malic dehydrogenase. Curve (2): 3.3×10^{-6} M DPNH. Curve (3): 3.3×10^{-6} M DPNH plus 3.6×10^4 units of malic dehydrogenase. Curve (4): 3.3×10^{-6} M DPNH, 3.6×10^4 units of malic dehydrogenase, and 5×10^{-2} M D-malate. Fluorescence was measured at the appropriate emission maximum for each system. Buffer, 0.1 μ phosphate, pH 7.15, containing 10^{-3} M EDTA. Final volume, 2 ml. Temp., 23.5°C.

coenzyme complexes are qualitatively similar to those observed with liver alcohol dehydrogenase [8]. The ratio $K_{E,O}/K_{E,R}$ decreases from about 1 400 at pH 6.0 to 100 at pH 8.0. $K_{E,O}$ decreases sharply from pH 6.0 to 7.15, indicating that a group with a pK in this region is involved in the binding of DPN. The mutual stabilization in the binding of D-malate and DPNH is also observed at pH 6 and 8, although $K_{EI,R}$, $K_{E,R}$, $K_{ER,I}$ and $K_{E,I}$ each increase with increasing pH. These studies are being completed and extended and a detailed report will be published.

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