# Thermodynamic Studies of Binary and Ternary Complexes of Pig Heart Lactate Dehydrogenase†

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ABSTRACT: The thermodynamics of the reaction catalyzed by pig heart muscle lactate dehydrogenase (LDH; EC 1.1.1.27) have been studied in 0.2 M potassium phosphate buffer, pH 7, over the temperature range of 10 to 35 °C by using oxamate and oxalate to simulate the corresponding reactions of the substrates pyruvate and lactate, respectively. The various complexes formed are characterized by Gibbs free energies, enthalpies, and entropies. The Gibbs free energies were determined by equilibrium dialysis investigations, fluorescence titrations, and ultraviolet difference spectroscopy, while the reaction enthalpies stem from direct calorimetric measurements. Formulas are given for both the temperature dependence of the equilibrium constants and the variation with temperature of the enthalpies involved in the four reactions between LDH and NADH or NAD, LDH–NADH and oxamate, and LDH–NAD and oxalate. All reactions show a marked negative temperature coefficient, $\Delta c_p$, of the binding enthalpies indicating partial refolding to be associated with binary and ternary complex formation. This interpretation appears very probable in view of recent x-ray crystallographic studies on lactate dehydrogenase from dogfish, which demonstrate a volume decrease to occur on binding of oxamate to the LDH–NADH complex. The validity of the thermodynamic parameters, as derived with substrate analogues, for the actual catalytic reaction, gains strong support from the agreement between the sum of the heats involved in the four intermediary reactions reported in this study and direct determinations of the overall enthalpy associated with the catalytic process published in the literature.

Pig heart muscle lactate dehydrogenase (LDH[1]) has long been the subject of extensive chemical and physical studies. The wealth of information on this isozyme and on the class of lactate dehydrogenases has been soundly covered by recent review articles (Sund, 1970; Holbrook and Gutfreund, 1973; Everse and Kaplan, 1973; Holbrook et al., 1975). This renders it possible to confine ourselves to cite some investigations pertinent to the execution and discussion of this study. An obligatory, sequential mechanism of the catalytic reaction of LDH characterized by binding of the coenzyme prior to the substrate has been established by steady-state kinetic investigations (Takenaka and Schwert, 1956, Zewe and Fromm, 1965) and equilibrium reaction rate measurements (Silverstein and Boyer, 1964). The existence and the rates of formation of individual complexes in the reaction sequence have been demonstrated by rapid reaction techniques (Whitaker et al., 1974; Südi, 1974). The primary structure of the skeletal muscle isozyme from dogfish has been determined (Taylor et al., 1973) and partial sequences of lactate dehydrogenases from different species have been shown to comprise large homologies (Steginkt et al., 1971; Mella et al., 1969; Pfleiderer et al., 1970; Torff et al., to be published). In particular, a peptide containing the essential histidine residue of the active center of pig heart muscle lactate dehydrogenase has been isolated (Woenckhaus et al., 1969), which is in very close agreement with residues 191–203 in the dogfish catalytic domain. The available structural information has been completed by the detailed x-ray analysis of the apoenzyme and the various enzyme–coenzyme and enzyme–coenzyme–inhibitor complexes of LDH from dogfish ($M_4$ type) at high resolution (2.0–3.0 Å) (Adams et al., 1970, 1972, 1973); preliminary electron density maps of pig heart muscle LDH complexed with NADH and oxamate have been obtained with a nominal resolution of 6.0 Å (Holbrook et al., 1975). The detailed knowledge of structural and kinetic properties of lactate dehydrogenases is contrasted by the fragmentary information on the thermodynamics of the various interactions between the enzyme, the oxidized or reduced coenzyme, and the different ligands. Therefore extensive calorimetric studies in connection with fluorescence, uv-absorption, and equilibrium dialysis experiments have been performed to establish Gibbs free energies, enthalpy, and entropy values for the reactions between pig heart muscle LDH, NADH or NAD, and the substrate analogous inhibitors oxamate and oxalate, in order to provide a quantitative basis for the discussion of the energetic contributions involved in the individual steps of the catalytic reaction.

## Experimental Section

### *Materials*

Pig heart muscle LDH, NADH, and NAD were purchased from Boehringer, Mannheim, Germany. LDH was supplied as a 1% crystalline suspension in ammonium sulfate. Reagent grade potassium phosphate and potassium oxalate were obtained from E. Merck, Darmstadt, Germany; sodium oxamate was an Aldrich product. Demineralized and quartz distilled water was used for the buffer solutions. The enzyme crystals were collected from the suspension by centrifugation (40 000*g*, 15 min), dialyzed for 24 h at 4 °C under nitrogen against the buffer solution, with three changes of the buffer thousand times the volume of the enzyme solution. The resulting enzyme solutions had activities between 410 and 450 units/mg in the standard test (Biochemica Informationen, Boehringer). Enzyme concentration was determined using an absorption

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[1] Abbreviations used: LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adenine dinucleotide.

coefficient $A = 1.4$ cm$^2$ mg$^{-1}$ at 280 nm. The ratio of the absorption of the enzyme at 280 nm to that at 260 nm was 1.98. This ratio, the activity, and fluorescence spectra served as criteria of purity of the enzyme, guaranteeing a uniform quality when enzyme preparations from different lots were used. Coenzymes and substrate analogues were dissolved in the buffer resulting from the last dialysis. Concentrations of the coenzymes were determined spectrophotometrically, using the following absorption coefficients: NAD, $18.0 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 260 nm; NADH, $6.23 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 340 nm (Beisenherz et al., 1955); oxamate and oxalate concentrations were based on weight.

### *Methods*

*Binary Complex Formation.* Dissociation constants of the binary complexes between LDH and NADH were determined by equilibrium dialysis experiments. A self-constructed unit was employed accommodating 48 dialysis cells (Myer and Schellman, 1962) with a volume of 0.25 cm$^3$ each. Measurements were performed on enzyme solutions identical in concentration with the samples used for the calorimetric determinations (approximately 20 $\mu$M). Concentrations of free coenzyme were determined fluorimetrically.

Binding of the oxidized coenzyme to the apoenzyme was studied by the same method, the concentration of the free NAD being determined by uv absorption. Enzyme concentration was also approximately 20 $\mu$M.

Generally equilibrium was reached according to temperature within 5 to 12 h and assured by the coincidence of equilibrium constants derived from dialysis experiments starting both with a mixture of coenzyme and enzyme and the separated components (Steinhardt and Reynolds, 1969; Cassel et al., 1969).

Calorimetric experiments were carried out using an LKB batch microcalorimeter. One-milliliter samples of apoenzyme (~20 $\mu$M) and coenzyme solutions were mixed and the resulting heats were corrected for the effects due to dilution and friction by blank experiments. The coenzyme concentrations employed in the measurements with NADH were such as to achieve saturation of the protein; for the measurements with NAD, the degree of saturation has been calculated on the basis of the equilibrium constant at 25 °C, $K_{D,NAD} = 325$ $\mu$M.

*Ternary Complex Formation.* Reaction of oxamate with the LDH–NADH complex (10–15 $\mu$M LDH concentration) was followed by fluorescence measurements, exploiting fluorescence quenching resulting from addition of oxamate to the binary complex (Winer and Schwert, 1959). Excitation and emission wavelengths were 320 and 435 nm, respectively. The equilibrium constants were derived from titration curves employing common linearization procedures (Stinson and Holbrook, 1973).

Binding constants for the reaction of oxalate with the enzyme in the presence of the oxidized coenzyme were determined by uv difference spectroscopy in the wavelength range of 270 to 320 nm, the detailed procedures being published in a separate communication (Schmid et al., 1976).

A technique analogous to that applied to binary complex formation was adopted for determination of the enthalpies involved in the reactions of oxamate and oxalate with the LDH–NADH and the LDH–NAD complexes, respectively. Samples of 1 ml of enzyme solution containing an excess of the reduced or oxidized coenzyme were mixed with the same volume of the appropriate substrate analogue dissolved in the buffer having an identical coenzyme concentration. Again, the necessary corrections of the measured heats were provided by blank experiments.

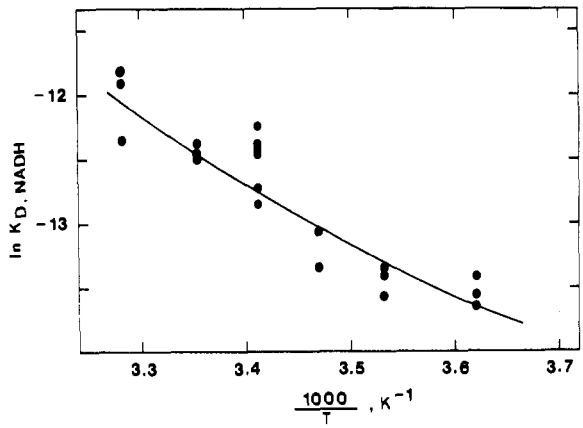



FIGURE 1: Van't Hoff plot of the equilibrium constant for the dissociation of NADH from pig heart muscle lactate dehydrogenase (0.2 M potassium phosphate buffer, pH 7, enzyme concentration range, 17.5–19.0 $\mu$M). The points refer to the experimental results, while the curve has been calculated on the basis of eq 1.

Absorption measurements were performed in a Zeiss DMR 10 double-beam scanning spectrophotometer; fluorescence was determined employing an Hitachi Perkin-Elmer MPF 2 A fluorimeter. Temperature of the cell compartments of either spectrophotometer was controlled by Haake KT 33 thermostats and measured in the cells using a YSI Model 46 TUC Tele-Thermometer. pH values of the buffer solutions were adjusted by means of a WTW pH meter (Digi 510, Wiss-Tech. Werkstätten, Weilheim, Germany).

## Results

### *Binary Complexes*

*Temperature Dependence of the Equilibrium Constants for Binary Complex Formation between LDH and NADH or NAD.* Figure 1 summarizes in a van't Hoff plot the results obtained by equilibrium dialysis in 0.2 M potassium phosphate buffer, pH 7, and enzyme concentrations between 17.5 and 19.0 $\mu$M for the reaction between LDH and NADH. The experimental points are well represented by the curve, which is the graph of eq 1, calculated on the basis of the equilibrium constant at 25 °C, $K_{D,NADH} = 3.9$ $\mu$M, and the calorimetrically determined enthalpies, using the Gibbs–Helmholtz equation:

$$\ln K_{D,NADH} = \frac{19\,673.635}{T} + 83.920 \ln T - 556.582 \quad (1)$$

$T$ refers to degrees kelvin. The number of digits to the right of the decimal point has been retained with the consideration that the equilibrium constant at 25 °C should be reproduced, when applying eq 1 to $T = 298.15$ K. The view was adopted for the analogous equations (eq 2, 5, and 7).

Due to the excellent accordance of the experimental and calculated results for the association of LDH and NADH, determination of the binding constant by equilibrium dialysis for the reaction between LDH and NAD was confined to 25 °C, as reported in Table I. Temperature variation of the equilibrium constant for this reaction was obtained following the same procedure as mentioned above with the result given by eq 2.

$$\ln K_{D,NAD} = \frac{9460.132}{T} + 42.023 \ln T - 279.192 \quad (2)$$

TABLE I: Equilibrium Constant for the Dissociation of NAD from Pig Heart Muscle Lactate Dehydrogenase as Determined by Equilibrium Dialysis at 25 °C in 0.2 M Potassium Phosphate Buffer (pH 7).

| $[E]_0$[a] (μM) | $N$[b] | $K_{D,NAD} \times 10^4$[c] (M) |
|---|---|---|
| 66.1 | 4 | 3.2 ± 0.4 |
| 85.5 | 6 | 3.2 ± 0.4 |
| 85.5 | 6 | 3.3 ± 0.2 |

[a] Total concentration of binding sites. [b] Number of experiments. [c] Dissociation constant ± standard error.

TABLE II: Temperature Dependence of the Enthalpy of Dissociation of NADH from Pig Heart Muscle Lactate Dehydrogenase (0.2 M Potassium Phosphate Buffer, pH 7).

| $\vartheta$ (°C) | $[E]_0$[a] (μM) | $[NADH]_0$[b] (μM) | $\Delta H_{D,NADH}$[c] (kcal/mol of LDH) |
|---|---|---|---|
| 10 | 21.3 | 1027 | 32.9 |
| 10 | 19.2 | 975 | 32.9 |
| 10 | 19.1 | 1089 | 32.5 |
| 15 | 19.0 | 1096 | 36.4 |
| 15 | 19.0 | 1350 | 35.0 |
| 15 | 19.0 | 1365 | 35.7 |
| 20 | 19.2 | 1105 | 38.7 |
| 20 | 19.0 | 931 | 37.1 |
| 20 | 19.0 | 933 | 40.1 |
| 25 | 19.0 | 932 | 42.4 |
| 25 | 19.0 | 1049 | 43.1 |
| 30 | 19.0 | 1440 | 44.9 |
| 30 | 19.0 | 1720 | 43.3 |
| 30 | 19.1 | 1509 | 47.1 |
| 35 | 19.0 | 1588 | 49.7 |
| 35 | 19.0 | 1620 | 51.1 |

[a] Total enzyme concentration. [b] Total NADH concentration. [c] Heats of dissociation per mol of enzyme, i.e., per 4 mol of binding sites.

TABLE III: Temperature Dependence of the Enthalpy Involved in Dissociation of NAD from Pig Heart Muscle Lactate Dehydrogenase (0.2 M Potassium Phosphate Buffer, pH 7).

| $\vartheta$ (°C) | $[E]_0$[a] (μM) | $[NAD]_0$[b] (μM) | % Saturation[c] | $\Delta H_{D,NAD}$[d] (kcal/mol of LDH) |
|---|---|---|---|---|
| 10 | 19.0 | 3300 | 95.7 | 19.6 |
| 10 | 19.0 | 3300 | 95.7 | 19.8 |
| 17.5 | 19.0 | 3180 | 96.0 | 21.6 |
| 25 | 19.0 | 3300 | 91.5 | 24.5 |
| 25 | 19.0 | 3300 | 91.5 | 23.2 |
| 35 | 19.4 | 3300 | 87.8 | 27.4 |
| 35 | 19.4 | 3300 | 87.8 | 27.8 |

[a] Total enzyme concentration. [b] Total NAD concentration. [c] Calculated using the equilibrium constant at the various temperatures as derived from eq 2 and the mass law expression. [d] Heat of dissociation per mol of enzyme, i.e., per 4 mol of binding sites.

*Temperature Dependence of the Enthalpies of the Reactions between LDH and NADH or NAD.* The calorimetrically determined enthalpies involved in the interaction between LDH and the coenzyme in its reduced or oxidized form are shown in Tables II and III. The high association constant for the reaction of NADH and LDH rendered it possible to determine the heats of binding under essentially saturating conditions of the coenzyme, while the two orders of magnitude lower equilibrium constant for complex formation between LDH and NAD required measurements employing a degree of saturation lower than 100%. The corresponding percentage of binding sites occupied by NAD under the experimental conditions are listed in column 4 of Table III. The variation of the molar enthalpies of dissociation can be adequately represented by eq 3 and 4, which result from linear regression analyses of the data and refer to the reactions of LDH with NADH (eq 3) and NAD (eq 4).

$$\Delta H_{D,NADH} = 667\vartheta + 25\,575 \text{ cal mol}^{-1} \quad (3)$$

$$\Delta H_{D,NAD} = 334\vartheta + 16\,043 \text{ cal mol}^{-1} \quad (4)$$

$\vartheta$ indicates temperature in degrees centigrade. The standard errors of the estimates of the $\Delta H$ values are 1190 cal/mol (eq 3) and 773 cal/mol (eq 4), respectively.

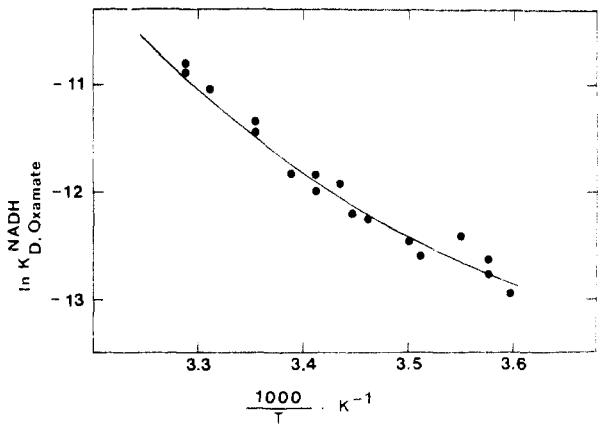



FIGURE 2: Van't Hoff plot of the equilibrium constant for the dissociation of oxamate from the binary LDH–NADH complex (0.2 M potassium phosphate buffer, pH 7, enzyme concentration, 5 μM). The points represent experimental data, while the curve has been calculated using eq 5.

TABLE IV: Temperature Dependence of the Enthalpies Involved in the Dissociation of Oxamate from the Binary LDH–NADH Complex (0.2 M Potassium Phosphate Buffer, pH 7).

| $\vartheta$ (°C) | $[E]_0$[a] (μM) | $[Oxamate]_0$[b] (mM) | $\Delta H_{D,oxamate}^{NADH}$[c] (kcal mol$^{-1}$) |
|---|---|---|---|
| 10 | 19.0 | 17.4 | 35.8 |
| 10 | 10.0 | 24.5 | 37.5 |
| 11 | 18.9 | 15.4 | 34.1 |
| 15 | 18.9 | 11.7 | 45.0 |
| 20 | 10.0 | 24.8 | 51.4 |
| 25 | 20.1 | 5.9 | 64.4 |
| 25 | 19.3 | 10.3 | 61.6 |
| 25 | 19.0 | 10.8 | 60.8 |
| 30 | 10.1 | 18.3 | 66.2 |
| 30 | 10.1 | 18.2 | 69.2 |
| 30 | 10.0 | 21.4 | 67.6 |
| 35 | 10.0 | 33.4 | 73.4 |
| 35 | 10.0 | 29.6 | 76.9 |
| 35 | 10.1 | 22.4 | 77.8 |

[a] Total enzyme concentration. [b] Total oxamate concentration. [c] Dissociation enthalpy per mol of enzyme, i.e., per 4 mol of binding sites.

TABLE V: Temperature Dependence of the Enthalpy Involved in the Dissociation of the Ternary Complex LDH-NAD-Oxalate into the Constituents (0.2 M Potassium Phosphate Buffer, pH 7).

| $\vartheta$ (°C) | $[E]_0$[a] ($\mu$M) | $[NAD]_0$[b] ($\mu$M) | $[Oxalate]_0$[c] (mM) | $\Delta H_{D,NAD,ox}$[d] (kcal mol$^{-1}$) | $\Delta H_{D,ox}^{NAD}$[e] (kcal mol$^{-1}$) |
|---|---|---|---|---|---|
| 10 | 10.0 | 580 | 26.0 | 31.7 | 12.3 |
| 10 | 10.0 | 680 | 22.0 | 29.6 | 10.2 |
| 10 | 10.0 | 680 | 22.0 | 30.8 | 11.4 |
| 25 | 19.1 | 1600 | 18.2 | 55.4 | 31.0 |
| 25 | 14.7 | 975 | 11.2 | 56.0 | 31.6 |
| 25 | 19.0 | 870 | 10.6 | 53.3 | 28.9 |
| 35 | 10.0 | 650 | 10.6 | 71.6 | 43.9 |
| 35 | 9.9 | 345 | 2.9 | 73.5 | 45.8 |
| 35 | 9.9 | 340 | 11.6 | 74.1 | 46.4 |

[a] Total enzyme concentration. [b] Total NAD concentration. [c] Total oxalate concentration. [d] Heat of dissociation of the ternary complex into LDH, NAD, and oxalate per mole of enzyme. [e] Enthalpy involved in dissociation of oxalate from the LDH-NAD complex per mole of enzyme. The value is obtained by subtracting from the overall enthalpy, given in column 5, the heat resulting from the dissociation of the binary complex, LDH-NAD, as calculated using eq 4.

*Ternary Complexes*

*Temperature Dependence of the Equilibrium Constant for the Interaction between the Binary LDH-NADH Complex and Oxamate.* The dependence on temperature of the dissociation constant, $K_{D,oxamate}^{NADH}$, for the reaction of oxamate and LDH in the presence of NADH in 0.2 M potassium phosphate buffer (pH 7) is illustrated by the van't Hoff plot shown in Figure 2. Data were derived from titration curves employing the quenching of coenzyme fluorescence (excitation at 320 nm, emission at 435 nm) in the course of increasing saturation of the LDH-NADH complex with oxamate. There is again good agreement between the experimentally determined temperature variation and the theoretical curve, given by eq 5

$$\ln K_{D,oxamate}^{NADH} = \frac{53\,164.143}{T} + 203.825 \ln T - 1351.125 \quad (5)$$

which is based on the dissociation constant at 20 °C and the calorimetric $\Delta H$ values.

*Temperature Dependence of the Enthalpies for the Reaction between the LDH-NADH Complex and Oxamate.* Calorimetric studies on the heats involved in the interaction between oxamate and the binary LDH-NADH complex resulted in the enthalpy values summarized in Table IV. Analysis of the variation of the dissociation enthalpies with temperature yields a linear function, given by eq 6

$$\Delta H_{D,oxamate}^{NADH} = 1620\vartheta + 19\,814 \text{ cal mol}^{-1} \quad (6)$$

$\vartheta$ refers to degrees centigrade and the enthalpy is calculated per mol of enzyme with a standard error of 2091 cal mol$^{-1}$.

*Ternary Complex Formation between LDH-NAD and Oxalate: Equilibrium and Calorimetric Analyses.* The equilibrium constant for the association of the binary LDH-NAD complex and oxalate has been determined employing changes of the ultraviolet absorption spectrum in the range of 270 to 320 nm concomitant with binding of oxalate (Schmid et al., 1976).

At 25 °C, pH 7 in 0.2 M potassium phosphate buffer, a value for the dissociation constant $K_{D,oxalate}^{NAD}$ of 2.1 ± 0.1 $\mu$M was obtained. Temperature dependence of this equilibrium constant was again obtained by integration of the van't Hoff relationship, resulting in eq 7, with $T$ referring to the absolute temperature scale

$$\ln K_{D,oxalate}^{NAD} = \frac{46\,927.453}{T} + 170.609 \ln T - 1142.530 \quad (7)$$

A summary of the enthalpies determined calorimetrically at various temperatures in 0.2 M potassium phosphate buffer (pH 7) is shown in Table V. The $\Delta H_{D,NAD,oxalate}$ values refer to the overall heat involved in dissociating the ternary complex into the constituents LDH, NAD, and oxalate. The molar heat effects associated with the dissociation of oxalate from the binary LDH-NAD complex are assembled in column 6 of Table V. They are calculated on the basis of the additivity of enthalpies by subtracting the $\Delta H_{D,NAD}$ values as determined using eq 4 from the overall enthalpy $\Delta H_{D,NAD,oxalate}$ given in column 5 of Table V. A polynomial regression analysis of the first degree afforded eq 8 for the temperature variation of the molar dissociation enthalpy $\Delta H_{D,oxalate}^{NAD}$, with a standard error of the estimate of 1346 cal/mol.

$$\Delta H_{D,oxalate}^{NAD} = 1356\vartheta - 2588 \text{ cal mol}^{-1} \quad (8)$$

$\vartheta$ relates to degrees centigrade.

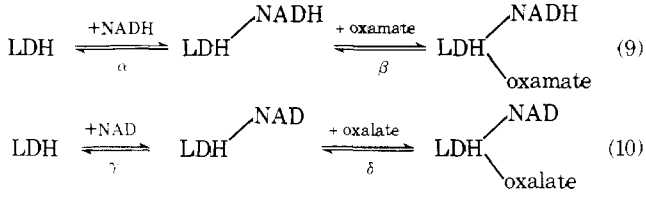
## Discussion

The substantial amount of information on the lactate dehydrogenase reaction chiefly pertains to data on the sequence and the tertiary and quaternary structure of the intermediate complexes of the enzyme with the various ligands as well as to the kinetic mechanism of these reactions. With a few exceptions, which will be discussed later, thermodynamic data have been solely represented by equilibrium constants, i.e., by free energy values. However, for a complete thermodynamic characterization, which is appropriate for a discussion of the thermodynamic properties in molecular terms, a correlation of the free energy data with reaction enthalpies is desirable.

In order to provide a self-consistent set of apparent $\Delta G$, $\Delta H$, and $\Delta S$ values, delineated under identical experimental conditions, equilibrium constants have been reevaluated despite the existence of single analogous values since usually one or several of the parameters, such as pH, buffer, temperature, or enzyme concentration, differed somewhat from the experimental conditions applied to the calorimetric investigations performed in this study. Our choice to use a 0.2 M potassium phosphate buffer had originally been biased by a report (Vesell et al., 1968) arriving at the result that heart as well as skeletal muscle LDH from rat exhibits maximum activity and stability

in that buffer, two properties important for calorimetric measurements of relatively long duration. In the meantime calorimetric studies on the pig skeletal muscle LDH have been performed (Hinz and Jaenicke, 1973, 1975) requiring identity of the solution parameters for a quantitative comparison of the thermodynamic values of the two isozymes.

Thermodynamic analysis of the various interactions of LDH with the ligands followed the general scheme of an ordered mechanism with coenzyme binding preceding the formation of ternary complexes with the substrates. However, since direct calorimetric experiments with the substrates pyruvate and lactate, respectively, are not feasible due to the catalytic reaction, measurements have been carried out with the substrate analogous inhibitors oxamate and oxalate (Novoa et al., 1959; Winer and Schwert, 1959) which have also been used in the x-ray crystallographic studies (Adams et al., 1972, 1973). Thus the two sequences assumed to provide a reasonable representation of the actual catalytic process and therefore a basis for an interpretation of the thermodynamic data, pertinent to the lactate dehydrogenase reaction, are illustrated in the following scheme.




The letters refer to the four reactions chosen to simulate the lactate dehydrogenase mechanism. Steps $\alpha$ and $\beta$ are to represent the analogous intermediates of the enzyme with the reduced coenzyme followed by binding of pyruvate, while sequence 10 corresponds to the association of LDH with the oxidized coenzyme and the subsequent ternary complex formation with lactate.

### *Thermodynamic Parameters for Binary Complex Formation*

*Equilibrium Data for the Reaction between LDH and NADH or NAD.* The van't Hoff plot in Figure 1 for the temperature dependence of the LDH–NADH equilibrium exhibits one of the rare examples within the field of enzyme-catalyzed processes and reactions involving biopolymers in general of good consistency between enthalpies obtained by direct calorimetric measurements and results which would be derived from a van't Hoff plot. Usually the variation with temperature of the reaction enthalpy evades detection by methods other than calorimetric techniques. The absolute values of the dissociation constants are in fair agreement with results published taking the different experimental conditions into account (Winer et al., 1959; D'A Heck, 1969; Stinson and Holbrook, 1973).

The dissociation constant, $K_{D,NAD}$, resulting from equilibrium dialysis experiments in 0.2 M potassium phosphate buffer (pH 7, at 25 °C), for the interaction between NAD and the enzyme of 325 $\mu$M is in good accordance with the value for 20 °C obtained by Stinson and Holbrook (1973) if one allows for enhanced dissociation due to the positive enthalpy of dissociation. However, the exact delineation of the dependence of $K_{D,NAD}$ on temperature (eq 2) was possible on the basis of the calorimetric results, which rendered it possible to avoid equilibrium dialysis studies or kinetic competition experiments as a function of temperature, both of which are rather cumbersome for binary complex formation between LDH and NAD, regarding the weak affinity for NAD and the lack of easily detectable changes in physical properties associated with binding.

*Calorimetric Data for the Reaction between LDH and NADH or NAD.* Calorimetric analysis of the interaction between LDH and the reduced or oxidized coenzyme as a function of temperature resulted in the enthalpies given in Tables II and III. Within the limits of experimental uncertainty, the variation with temperature of $\Delta H_{D,NADH}$ as well as that of $\Delta H_{D,NAD}$ can be represented by linear functions as demonstrated by eq 3 and 4. The temperature coefficient per mol of binding site, $\Delta c_p = -167$ cal (mol of binding site)$^{-1}$ deg$^{-1}$ of the enthalpy involved in association of LDH and NADH is by a factor of 2 more negative than the corresponding value for the interaction with NAD, $\Delta c_p = -83.5$ cal (mol of binding site)$^{-1}$ deg$^{-1}$, as can be seen in column 6 of Table VI (reactions $\alpha$ and $\gamma$).

Comparing the magnitude of the heats involved in binding of NADH to the pig heart muscle isozyme with those recently obtained for the same reaction with the pig skeletal muscle isozyme, one recognizes that the $\Delta H$ values for the reaction of NADH with the pig skeletal muscle isozyme are smaller in the whole temperature range studied (Hinz and Jaenicke, 1975). However, the temperature coefficient of the binding enthalpy per site is by almost a factor of 2 larger for the pig skeletal muscle isozyme ($\Delta c_p = -325$ cal (mol of binding site)$^{-1}$ deg$^{-1}$) compared with the pig heart muscle isozyme ($\Delta c_p = -167$ cal (mol of binding site)$^{-1}$ deg$^{-1}$).

In view of accepted interpretations of the temperature coefficient of $\Delta H$ as a heat capacity change induced by binding of the ligand (in this case the coenzyme), the different magnitudes of the $\Delta c_p$ values can be assumed to reflect distinct variations in the degree of structural changes associated with the binding reactions; the structural changes may consist of a reduction in the exposure of hydrophobic groups to the solvent and (or) a decrease of the number of excitable degrees of freedom with respect to rotational and vibrational modes of the system. Presently, it is still left to future understanding of the water structure with its implications concerning hydrophobic interaction to fully explain the macroscopic thermodynamic quantities by molecular models. However, there is strong evidence that a negative heat capacity change indicates a "tightening" of the macromolecular structure, which means, for the reaction of LDH with either coenzyme, that partial "refolding" occurs on binding, the effect being much more pronounced with NADH than with NAD (Velick et al., 1971; Tanford, 1968; Hearn et al., 1971; Hinz et al., 1971; Hinz and Jaenicke, 1975). This result is at variance with the discussion of thermodynamic parameters of the beef heart LDH reaction, given by Borgmann et al. (1974). These authors performed kinetic experiments, leading to the determination of eight individual rate constants, at 0 and 50 °C. On the basis of the ratio of the velocity constants for the association and dissociation reactions and the activation data resulting from experiments at two temperatures, they calculated Gibbs free energies, reaction enthalpies, and entropies. Although a comparison of the results remains ambiguous in so far as the kinetic measurements of Borgmann et al. were performed on the beef heart muscle isozyme, while the thermodynamic investigations reported in this study employed pig heart muscle LDH, it is difficult to rationalize the occurrence of dramatic alterations like a change in sign of the reaction entropy for NADH binding, if one takes the well-documented interspecies similarity of LDH isozymes into account. It should be noted, however, that there has been a similar report on the kinetics of the beef

TABLE VI: Apparent Thermodynamic Parameters for the Lactate Dehydrogenase Reaction at 25 °C in 0.2 M Potassium Phosphate Buffer (pH 7).[a]

| Reaction | $10^{-3}K_A$[b] | $\Delta G_A$[c] | $\Delta H_A$[d] | $\Delta S_A$[e] | $\Delta c_{p_A}$[f] |
|---|---|---|---|---|---|
| $\alpha$ | 260 ± 30 | −7.39 ± 0.08 | −10.6 ± 0.3 | −10.8 ± 1.3 | −167 ± 9 |
| $\beta$ | 86 ± 9 | −6.73 ± 0.06 | −15.1 ± 0.5 | −28.0 ± 1.9 | −405 ± 21 |
| $\gamma$ | 3.08 ± 0.18 | −4.76 ± 0.04 | −6.1 ± 0.2 | −4.5 ± 0.8 | −84 ± 8 |
| $\delta$ | 476 ± 24 | −7.74 ± 0.03 | −7.8 ± 0.3 | 0 ± 0.1 | −339 ± 11 |

[a] The binding reactions of pyruvate and lactate have been simulated by oxamate and oxalate, respectively. The thermodynamic values refer to the reactions specified in column 1 by the letters $\alpha$ to $\delta$, which correspond to the reaction scheme in the Discussion section. [b] Association constants ± standard errors (in units of $M^{-1}$). [c] In kcal (mol of binding site)$^{-1}$; Gibbs free energies of binding per mol of binding site. The error limits correspond to the uncertainty of the binding constants in column 2. [d] In kcal (mol of binding site)$^{-1}$; enthalpies per mol of binding site ± standard error calculated using eq 3, 6, 4, and 8. [e] In cal deg$^{-1}$ (mol of binding site)$^{-1}$; reaction entropies per mol of binding site. The error limits relate to the situation when the uncertainties of the $\Delta H_A$- and $\Delta G_A$ values go into opposite direction. [f] In cal deg$^{-1}$ (mol of binding site)$^{-1}$; heat capacity changes per mol of binding site involved in the association reaction. Error limits have been derived from linear regression analyses.

heart LDH by Takenaka and Schwert (1956) in which the dissociation constants for both coenzymes, obtained as the ratio of complex kinetic constants, were found to exhibit a negative temperature coefficient, i.e., dissociation from the binary complex decreased with increasing temperature.

Considering the relatively large uncertainties involved in estimating thermodynamic parameters on the mere basis of kinetic evidence, equilibrium data and calorimetrically determined $\Delta H$ values as well as their directly measured temperature dependence appear to constitute a more solid foundation for the interpretation of reaction enthalpies and entropies.

*Comparison of the Enthalpies for Binary Complex Formation with Previous Results.* The only estimate of the enthalpy for the interaction of pig heart muscle LDH and NADH (67 mM sodium phosphate buffer (pH 7.2, 20 °C)) has been reported by Stinson and Holbrook (1973), amounting to $\Delta H = -12.4$ kcal/mol of NADH for the binding process. This value results from an apparently linear van't Hoff plot and is therefore supposed to be temperature independent. The magnitude of the enthalpy shows moderately good agreement with the value of $\Delta H_{A,NADH} = -10.6$ kcal/mol of NADH at 25 °C determined calorimetrically (Table VI, reaction $\alpha$, column 4). However, in contrast to the results displayed in Figure 1 and Table II of this study, the variation of $\Delta H$ with temperature remained undetected.

### *Thermodynamic Parameters for Ternary Complex Formation*

*Equilibrium and Calorimetric Analysis of the Reaction between the Binary LDH-NADH Complex and Oxamate.* A summary of the equilibrium constants as obtained by fluorescence measurements in 0.2 M potassium phosphate buffer, pH 7, at various temperatures is given by the van't Hoff plot in Figure 2. The calorimetric results and the data derived from the fluorescence titrations are again mutually consistent. The temperature variation of the enthalpy involved in the dissociation of oxamate from the LDH-NADH complex, as given by eq 6, is distinctly reflected in the curvature of the van't Hoff plot. The most prominent result apart from this accordance is the detection of the heat capacity change of $\Delta c_p = -405$ cal (mol of binding site)$^{-1}$ deg$^{-1}$ (Table VI, column 6, reaction $\beta$), accompanying the binding of oxamate to LDH in the presence of NADH. This value is more than twice as large as the heat capacity change concomitant with binary complex formation between LDH and NADH, indicating major structural changes toward further "folding" to occur, when the ternary complex is formed. These findings are corroborated considering the structural similarity of lactate dehydrogenases by results from the x-ray analysis of the LDH-NADH-oxamate complex of the dogfish enzyme, demonstrating a 6% decrease of the volume relative to the apoenzyme on complex formation (see review by Holbrook et al., 1975). It is not possible to make a direct assignment of $\Delta c_p$ to the percentage of structural change since the dogfish enzyme belongs to the $M_4$ type of lactate dehydrogenases which apparently is more labile with respect to conformational changes as judged by the significantly more negative $\Delta c_p$ obtained, when NADH binds to pig skeletal muscle enzyme ($M_4$ type) (Hinz and Jaenicke, 1975). However, the very fact that the calorimetric results not only show the existence but also the correct direction of a structural change in agreement with the x-ray analysis findings constitutes an excellent example for the usefulness of the calorimetric approach.

*Comparison with Results Reported in the Literature.* Thermodynamic parameters for the reaction of pig heart muscle LDH with oxamate in the presence of NADH have been obtained by D'A Heck (1969) employing fluorescence titrations and temperature-jump techniques. The corresponding thermodynamic association values resulting from experiments performed at 20 °C in 0.1 M sodium phosphate buffer, pH 7, with ionic strength of 0.3 M adjusted with NaCl were respectively: $\Delta G° = -6.54 \pm 0.20$ kcal (mol of binding site)$^{-1}$, $\Delta H° = -7.40 \pm 0.80$ kcal (mol of binding site)$^{-1}$, $\Delta S° = -2.9 \pm 3.4$ cal (mol of binding site)$^{-1}$ deg$^{-1}$. The main discrepancy between these values and those reported in Table VI, line 2, of this study arises from the marked difference in the magnitude of the $\Delta H$ value, even if one allows for the temperature difference of 5 °C, which would make the enthalpy value reported by D'A Heck about 2 kcal more negative, using the $\Delta c_p$ of −405 cal (mol of NADH)$^{-1}$ deg$^{-1}$. The reason for this disagreement is not clear.

The second report on the reaction enthalpy originates from Novoa et al. (1959), who found a temperature-independent $\Delta H$ value for the association of the LDH-NADH complex of beef heart muscle LDH and oxamate of $-6.9 \pm 0.3$ kcal/mol of NADH. The value has been calculated on the basis of the temperature variation of kinetically determined inhibition constants of oxamate, in 0.05 M phosphate buffer, pH 6.8. The origin of the difference in $\Delta H$ in comparison with the direct calorimetric values is again not obvious.

Another estimate for the enthalpy involved in association of pyruvate and the LDH-NADH complex has also been given by Novoa et al. They calculated this reaction to release 18.8

± 0.9 kcal/mol of binding site. This value compares favorably with $\Delta H_{D,oxamate}^{NADH} = -15.1$ kcal/mol of binding site, given in Table VI, column 4, for the oxamate reaction, suggesting that oxamate simulates the substrate pyruvate rather well with respect to ternary complex formation, a view also supported by the x-ray analysis (Adams et al., 1973).

*Equilibrium and Calorimetric Analysis of the Reaction between Oxalate and the LDH–NAD Complex.* As for the reaction between LDH and NAD, the temperature variation of the equilibrium constant for ternary complex formation with oxalate expressed by eq 7 results from integration of the van't Hoff relationship. The value of the dissociation constant at 25 °C, $K_{D,oxalate}^{NAD} = -2.1 \pm 0.1$ μM, obtained in 0.2 M potassium phosphate buffer (pH 7) for the pig heart muscle enzyme, is numerically identical with the value found by Novoa and Schwert (1961) for the same reaction in 0.05 M phosphate buffer, pH 7.4, at 23.1 °C, involving the beef heart muscle isozyme.

Inspection of the thermodynamic parameters for the interaction of oxalate with the LDH–NAD complex, shown in line 4 of Table VI, reveals that the association reaction is also characterized by a large negative $\Delta c_p$. This has to be expected since, assuming to a first approximation a common transition state for the LDH–NADH–pyruvate and LDH–NAD–lactate complexes in the catalytic reaction (Borgmann et al., 1974) and supposing further that oxamate and oxalate are reasonably good substitutes of the substrates pyruvate and lactate, respectively, it should be immaterial from which side of the reaction, NADH or NAD, the ternary complexes were formed.

The assumption that oxamate and oxalate are representative substrate analogues, in the sense that the thermodynamic parameters derived from their reactions constitute valid approximations for the actual catalytic process of pig heart muscle LDH, is supported by comparison with results for the overall enthalpy of the catalytic reaction. Direct calorimetric experiments on the reduction of pyruvate with NADH catalyzed by LDH in 0.15 M disodium phosphate buffer (pH 7.3, at 25 °C) resulted in a $\Delta H$ value of −10.6 kcal per mol of NAD produced (Katz, 1955). This value agrees well with the $\Delta H = -10.3 \pm 0.2$ kcal per mol of binding site arrived at by Hakala et al. (1956) when determining the variation of the overall equilibrium constant of pyruvate reduction with temperature.

Addition of the enthalpies given in column 4 of Table VI regarding the proper signs affords an overall $\Delta H$ value of −11.8 kcal per mol of NADH consumed, which is in good agreement with the enthalpies cited above considering the experimental uncertainties of the four independent calorimetric measurements.

The correspondence between the overall enthalpies appears to impart validity to the approach of elucidating thermodynamic details of the LDH reaction by use of appropriate substrate analogues.

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Dedication

This paper is dedicated to Professor Dr. Feodor Lynen on the occasion of his 65th birthday.

References

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Lentz, P. J., Jr., Rao, S. T., Rossmann, M. G., Smiley, I. E., and White, J. L. (1972), in Protein–Protein Interactions, Jaenicke, R., and Helmreich, E., Ed., Berlin, Springer-Verlag, pp 139–158.

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O., and Taylor, S. S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1968–1972.

Adams, M. J., Ford, G. C., Koekoek, R., Lentz, P. J., Jr., McPherson, A., Jr., Rossmann, M. G., Smiley, I. E., Schevitz, R. W., and Wonacott, A. J. (1970), *Nature (London) 227,* 1098–1103.

Beisenherz, G., Bücher, Th., and Garbade, K. H. (1955), *Methods Enzymol. 1,* 391–397.

Borgmann, U., Moon, Th. W., and Laidler, K. J. (1974), *Biochemistry 13,* 5152–5158.

Cassel, J., Gallagher, J., Reynolds, J. A., and Steinhardt, J. (1969), *Biochemistry 8,* 1706–1713.

D'A Heck, H. (1969), *J. Biol. Chem. 244,* 4375–4381.

Everse, J., and Kaplan, N. O. (1973), *Adv. Protein Chem. 37,* 61–133.

Hakala, M. T., Glaid, A. J., and Schwert, G. W. (1956), *J. Biol. Chem. 221,* 191–209.

Hearn, R. P., Richards, F. M., Sturtevant, J. M., and Watt, G. D. (1971), *Biochemistry 10,* 806–917.

Hinz, H.-J., and Jaenicke, R. (1973), *Biochem. Biophys. Res. Commun. 54,* 1432–1436.

Hinz, H.-J., and Jaenicke, R. (1975), *Biochemistry 14,* 24–27.

Hinz, H.-J., Shiao, D. F., and Sturtevant, J. M. (1971), *Biochemistry 10,* 1347–1352.

Holbrook, J. J., and Gutfreund, H. (1973), *FEBS Lett. 31,* 157–169.

Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975), *Enzymes, 3rd Ed., 11,* 191–292.

Katz, S. (1955), *Biochim. Biophys. Acta 17,* 226–232.

Mella, K., Torff, H.-J., Folsche, E. T. J., and Pfleiderer, G. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350,* 23–34.

Myer, Y. P., and Schellman, J. A. (1962), *Biochim. Biophys. Acta 55,* 361–373.

Novoa, W. B., and Schwert, G. W. (1961), *J. Biol. Chem. 236,* 2150–2153.

Novoa, W. B., Winer, A. D., Glaid, A. J., and Schwert, G. W. (1959), *J. Biol. Chem. 234,* 1143–1148.

Pfleiderer, G., Woenckhaus, C. J., Jeckel, D., and Mella, K. (1970), in Pyridine Nucleotide-Dependent Dehydrogenases, Sund, H., Ed., Berlin, Springer-Verlag, pp 145–156.

Schmid, F., Hinz, H.-J., and Jaenicke, R. (1976), *Hoppe-Seyler's Z. Physiol. Chem. 357,* 241–245.

Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochem. Biophys. 82,* 288–299.

Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239,* 3901–3907.

Stegink, L. D., Sanborn, B. M., Brummel, M. C., and Vestling, C. S. (1971), *Biochim. Biophys. Acta 251,* 31–37.

Steinhardt, J., and Reynolds, J. A. (1969), in Multiple Equilibria in Proteins, New York, N.Y., Academic Press.

Stinson, R. A., and Holbrook, J. J. (1973), *Biochem. J. 131,* 719–728.

Südi, J. (1974), *Biochem. J. 139,* 251–259.

Sund, H., Ed. (1970), Pyridine Nucleotide-Dependent Dehydrogenases, Berlin, Heidelberg, New York, Springer Verlag.

Takenaka, Y., and Schwert, G. W. (1956), *J. Biol. Chem. 223,* 157–170.

Tanford, C. (1968), *Adv. Protein Chem. 23,* 122–282.

Taylor, S. S., Oxley, S. S., Allison, W. S., and Kaplan, N. O.

(1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1790–1794.
Velick, S. F., Bagott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10,* 779–786.
Vesell, E. S., Fritz, P. J., and White, E. L. (1968), *Biochim. Biophys. Acta 159,* 236–243.
Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J., and Gutfreund, H. (1974), *Biochem. J. 139,* 677–697.
Winer, A. D., and Schwert, G. W. (1959), *J. Biol. Chem. 234,* 1155–1161.
Winer, A. D., Schwert, G. W., and Millar, D. B. S. (1959), *J. Biol. Chem. 234,* 1149–1154.
Woenckhaus, C., Berghäuser, J., and Pfleiderer, G. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 352,* 52–58.
Zewe, V., and Fromm, H. J. (1965), *Biochemistry 4,* 782–792.

# Mechanisms of Electron Transfer from Sulfite to Horseradish Peroxidase–Hydroperoxide Compounds†

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ABSTRACT: Using a rapid-scan spectrophotometer equipped with a stopped-flow apparatus, reactions of sulfite with compounds I and II of two horseradish peroxidase isoenzymes A and C were investigated. The direct two-electron reduction of peroxidase compound I by sulfite occurred at acidic pH but the mechanism gradually changed to the two-step reduction with the intermediate formation of compound II as the pH increased. The pH at which the one- and two-electron changes occurred at the same speed was 4.5 for peroxidase A and 7.7 for peroxidase C. A new peroxidase intermediate was found in the reaction between peroxidase compound II and sulfite. The sulfite compound showed a characteristic absorption band at 850 nm and the optical spectrum was similar to that of isoporphyrins but was quite different from that of sulfhemoproteins. The rate ($k$) of conversion from the sulfite–compound II complex to the sulfite compound was proportional to the concentration of $H^+$ and the log $k$ vs. pH plot for peroxidase A moved to the acidic side by 1.1 pH unit from that for peroxidase C.

It has been established by George (1952), Chance (1952a), and Yamazaki et al. (1960) that in the presence of hydrogen peroxide horseradish peroxidase catalyzes the one-electron oxidation of various bivalent redox molecules through the following catalytic cycle of the enzyme

$$\text{peroxidase} \xrightarrow{H_2O_2} \text{compound I} \xrightarrow{e^-} \text{compound II} \xrightarrow{e^-} \text{peroxidase} \quad (1)$$

On the other hand, Björkstén (1970) and Roman and Dunford (1972) have observed that there is a direct two-electron transfer from iodide to peroxidase compound I, thus forming the ferric enzyme without the intermediate formation of peroxidase compound II.

The definition and classification of one- and two-electron transfer mechanisms in enzymatic oxidation–reduction reactions have been reviewed by Yamazaki (1971). Various reactions between enzymes and substrates are grouped into two typical types: one- and two-electron transfers, while a few reactions are classified as a mixed type. The reaction of peroxidase compound I with sulfite is likely to belong to the mixed type (Roman and Dunford, 1973; Yamazaki and Yokota, 1973). This reaction appears to be suitable for studying the electron transfer mechanism on the basis of structure–function relation because a considerable amount of information has been given upon the structure of the catalytic site of horseradish peroxidase and the mechanism of electron transfer from sulfite to peroxidase compound I can be altered by the pH change.

In the course of the above experiment, a peculiar intermediate compound was found in the reaction of peroxidase compound II with sulfite. The detailed analysis of the reaction would also provide useful information concerning the relation between the mechanism of electron transfer and the structure of catalytic site of horseradish peroxidase.

## Materials and Methods

Horseradish peroxidase was purified from wild horseradish roots by the method of Shannon et al. (1966) with slight modification. Enzyme preparations used in this experiment were horseradish peroxidase C and horseradish peroxidase A, a mixture of isoenzymes $A_1$ and $A_2$, according to nomenclature by Paul (1958) and Shannon et al. (1966).

Buffer systems used were sodium acetate for pH 4.05–5.45, potassium phosphate for pH 6.00–7.60, and Tris-HCl for pH 8.01–8.40. The ionic strength in reaction solutions was adjusted to 0.1. Spectrophotometric measurements were performed with a Hitachi recording spectrophotometer, Model EPS-3T for ordinary time-scale experiments and with a Union Giken rapid reaction analyzer, RA-1300 for rapid reactions. The latter instrument combined with a flow apparatus can be used either as a rapid wavelength-scan spectrophotometer or as a sensitive photometer at a fixed wavelength. The absorption spectra are measured by means of a image dissector with a maximum speed of 300 nm/ms and are memorized in a digital computer system. The analogue replica is afterwards obtained in a X–Y recorder. The dead time of the flow apparatus at an $N_2$ gas pressure used in this experiment was less than 1 ms. For si-

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