tein kinase can be converted from a cAMP dependent form to a cAMP independent form. Efforts are currently being made in this laboratory to scale up the yield of purified cAMP dependent protein kinase. Direct structural studies on the isolated protein kinase in the presence of these perturbing agents will be under investigation.

Acknowledgments

We are indebted to James P. Charlton and Howard C. Froehlich for their dedicated technical assistance in the laboratory. We also extend thanks to Robert G. Langdon, Joseph Larner, and Carter M. Anderson, for comments regarding this manuscript.

References

Ackers, G. K. (1970), *Advan. Protein Chem. 24,* 343.
Andrews, P. (1970), *Methods Biochem. Anal. 18,* 1.
Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 24–44.
Brostrom, M. A., Reinmann, E. M., Walsh, D. A., and Krebs, E. G. (1970), *Advan. Enzyme Regul. 8,* 191.
Corbin, J. D., Brostrom, C. O., King, C. A., and Krebs, E. G. (1972), *J. Biol. Chem. 247,* 7790.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121,* 404.
Erlichman, J., Rubin, C. S., and Rosen, O. M. (1973), *J. Biol. Chem. 248,* 7607.
Gill, G. N., and Garren, L. D. (1970), *Biochem. Biophys. Res. Commun. 39,* 335.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Huang, L. C. (1974), *Biochim. Biophys. Acta 358,* 281.
Kumon, A., Yamamura, H., and Nishizuka, Y. (1970), *Biochem. Biophys. Res. Commun. 41,* 1290.
Langan, T. A. (1973), *Advan. Cyclic Nucl. Res. 3,* 94.
Laurent, T. C., and Killander, J. (1964), *J. Chromatogr. 14,* 317.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 261.
Sawyer, W. H., and Puckridge, J. (1973), *J. Biol. Chem. 248,* 8429.
Tao, M., Salas, M. L., and Lipmann, F. (1970), *Proc. Nat. Acad. Sci. U. S. 67,* 408.
Trotta, P. P., Pinkus, L. M., Haschemeyer, R. H., and Meister, A. (1974), *J. Biol. Chem. 249,* 492.
Vesterberg, O. (1971), *Methods Enzymol. 22,* 389.
Walsh, D. A., and Ashby, C. D. (1973), *Rec. Progr. Horm. Res. 29,* 329.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Winstead, J. A., and Wold, F. (1965), *Biochemistry 4,* 2145.
Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y. (1973), *Biochemistry 12,* 856.

Thermodynamics of Complex Formation between Nicotinamide Adenine Dinucleotide and Pig Skeletal Muscle Lactate Dehydrogenase†

Hans-Jürgen Hinz* and Rainer Jaenicke

ABSTRACT: Formation of the binary complex between the reduced coenzyme nicotinamide adenine dinucleotide (NADH) and pig skeletal muscle lactate dehydrogenase (LDH, EC 1.1.1.27) has been investigated by calorimetric and equilibrium dialysis techniques in 0.2 M potassium phosphate buffer (pH 7.0) at various temperatures. Analysis of thermal titration curves at two temperatures (25 and 31.5°) shows that the experimental enthalpy data can be rationalized assuming four independent and equivalent binding sites for the tetrameric enzyme. Binary complex formation is characterized by a negative temperature coefficient, Δc_p, of the binding enthalpy, which amounts to -1300 ± 53 cal/(deg mol of LDH) in the temperature range of 5–31.5°. Despite the slightly smaller standard deviation resulting when polynomial regression analysis of the second degree is applied to the temperature dependence of the enthalpy values, binding enthalpies seem to be adequately represented in the temperature range studied by the equation $\Delta H = -1.3T + 2.3$, kcal/mol of LDH, T referring to the temperature in °C. By combination of the results obtained from equilibrium dialysis and calorimetric studies a set of apparent thermodynamic parameters for binding of NADH to LDH in 0.2 M potassium phosphate buffer at pH 7 has been established.

Binding of the coenzyme NADH to lactate dehydrogenase (LDH) is associated with conformational changes of the complex as demonstrated by X-ray determinations (McPherson, 1970; Adams *et al.*, 1973). This direct experimental evidence supports results from earlier kinetic investigations which showed the binding reaction of coenzyme and apoenzyme to be best interpretable by a mechanism involving a fast diffusion controlled bimolecular step followed by a slower monomolecular interconversion (Czerlinski and Schreck, 1964). Unless compensatory effects occur, one should expect the conformational changes to be reflected in

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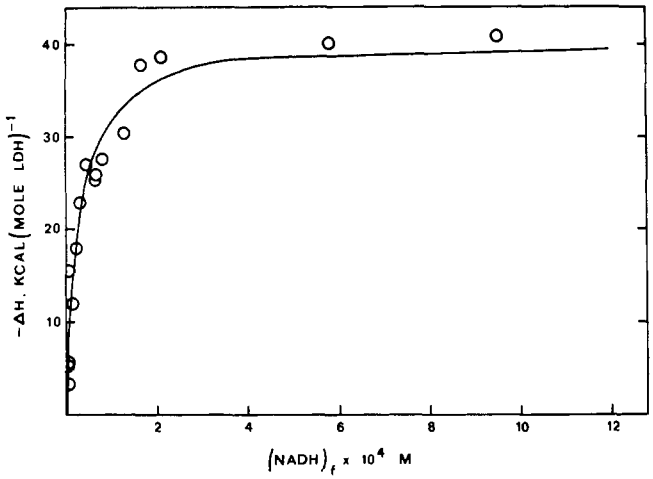

FIGURE 1: Thermal titration of pig skeletal muscle LDH with NADH at 31.5° in 0.2 M potassium phosphate buffer (pH 7). The heat absorbed when the coenzyme is dissociated is plotted as a function of free NADH concentration. Free NADH concentration is based on the equation: $\alpha = ((NADH)_f/K_D)/(1 + (NADH)_f/K_D)$, with α referring to the experimentally determinable degree of saturation of the enzyme and K_D denoting the dissociation constant. The curve is calculated on the assumption of a dissociation constant of 27.8 μM from the observed ΔH values (Table I) employing an enthalpy of 40.5 kcal/mole of LDH under saturation conditions.

the thermodynamic parameters of the reaction. Recent calorimetric investigations (Hinz and Jaenicke, 1973) provided values for the enthalpies of the reaction between LDH and NADH at 25°. These studies were extended to various temperatures, and supplemented by equilibrium dialysis experiments, in order to establish a complete set of thermodynamic data for the interaction between NADH and LDH.

Materials and Methods

Pig muscle lactate dehydrogenase and NADH disodium salt (grade I) were obtained from Boehringer, Mannheim Germany. LDH was supplied as a 1% crystalline suspension in ammonium sulfate and originated from different lots thus ensuring average properties with respect to preparation. All other chemicals employed were commercial preparations of reagent grade. The water used was demineralized and quartz distilled. The enzyme crystals were removed from the suspension by centrifugation (40,000*g*, 15 min) and dialyzed under a nitrogen atmosphere against the appropriate buffer solution at 4° for 24 hr, with three changes of a buffer 1000 times the volume of the enzyme solution. The resulting enzyme solutions had activities between 600 and 670 units/mg in the standard test (Biochemica Informationen, Boehringer). Activities were determined before and after the calorimetric experiment and at the time of the initiation of the calorimetric measurement with no changes detectable. The NADH solutions exhibited an E_{340}/E_{260} ratio of 0.40:0.41. The concentration of NADH was determined using a molar extinction coefficient $\epsilon = 6.22 \times 10^6$ cm^2/mol at 340 nm (Beisenherz *et al.*, 1955). Calorimetric measurements were made using a LKB batch microcalorimeter.

The equilibrium dialysis unit was constructed according to a model kindly provided to us by Dr. K. Kirschner. Concentration of free NADH was determined by fluorescence measurements employing a Hitachi-Perkin Elmer MPF 2A fluorimeter.

TABLE I: Enthalpy of Association, ΔH, of NADH with Pig Skeletal Muscle Lactate Dehydrogenase at pH 7.0 in 0.2 M Potassium Phosphate Buffer at Various Temperatures.

$[E]_0$[a] (μM)	$[NADH]_0$[b] (μM)	Temp (°C)	$-\Delta H$[c] (kcal/mol of LDH)
77.2	10.0	25	3.8
76.0	13.0	25	5.1
76.0	17.7	25	5.8
76.0	24.0	25	8.0
77.2	21.5	25	8.7
75.2	29.0	25	11.8
78.4	43.0	25	13.9
76.8	50.5	25	16.2
76.0	68.0	25	20.4
76.0	75.2	25	22.8
76.4	84.0	25	23.0
76.0	84.0	25	24.2
76.0	110.5	25	24.5
75.6	94.8	25	24.9
76.0	114.3	25	27.0
76.0	124.1	25	27.5
76.0	133.0	25	27.9
76.0	208.0	25	30.4
142.8	613.0	25	30.0
127.6	347.0	25	33.5
37.2	1206.7	25	29.2
21.2	845.3	25	27.6
76.0	1594.0	25	30.3
76.4	1471.1	25	27.6
76.0	11.3	31.5	3.2
76.4	11.3	31.5	5.4
75.6	13.0	31.5	5.5
76.4	36.5	31.5	11.9
76.0	32.8	31.5	15.3
76.0	55.1	31.5	17.9
76.4	75.9	31.5	22.7
60.4	102.0	31.5	25.1
76.0	113.5	31.5	25.9
76.4	95.3	31.5	26.9
76.4	133.1	31.5	27.7
76.0	186.4	31.5	30.4
75.6	238.0	31.5	37.8
76.4	286.0	31.5	38.7
144.0	722.4	31.5	40.1
144.0	1094.7	31.5	40.9
76.0	1234.1	5.3	4.0
76.0	1310.4	5.8	6.3
76.0	404.4	10.25	12.3
76.0	565.5	10.30	10.6
76.0	1564.0	10.75	14.5
76.0	1023.5	15.0	17.6
76.0	788.8	17.5	17.4
76.0	664.5	17.5	18.9
76.0	1063.3	20.0	22.2
76.0	132.0	20.0	24.2

[a] Total subunit concentration of the enzyme. [b] Total NADH concentration. [c] Enthalpy per mole of LDH (per four subunits).

Enzyme concentration is based on a molecular weight of 140,000 and an absorption coefficient $E_{0.1\%,1cm}$ 1.4 at 280 nm (Jaenicke and Knof, 1968).

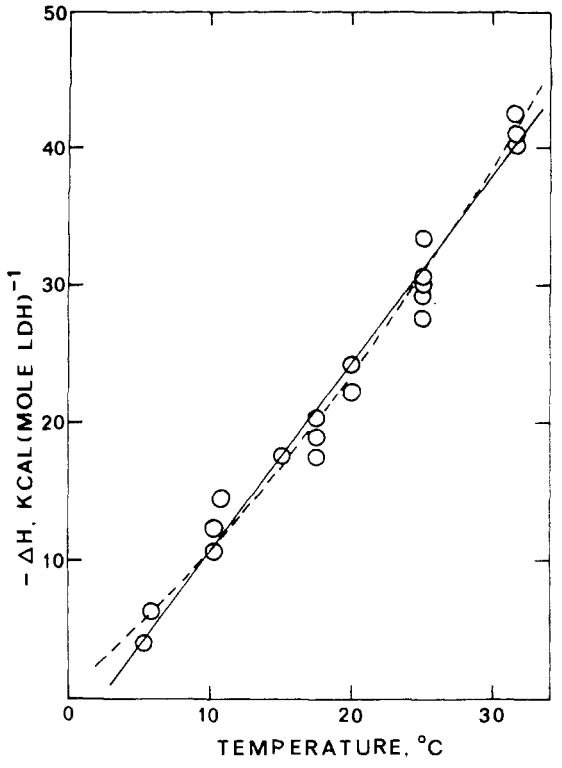

FIGURE 2: Variation of the molar enthalpy of dissociation of the complex between NADH and pig skeletal muscle LDH with temperature under saturating conditions of the coenzyme (0.2 M potassium phosphate buffer (pH 7). (—) Graphical representation of a linear regression analysis (eq 1); (- - -) results of a polynomial regression analysis of the second degree (eq 2).

Results

Enthalpy of Binding, ΔH, of NADH to Pig Muscle Lactate Dehydrogenase. The enthalpy accompanying the binding of NADH to LDH was determined as a function of coenzyme concentration at 31.5° (Figure 1) and under saturating conditions of the coenzyme at 5, 10, 15, 17.5, and 20° with the results shown in Table I. For comparison the numerical values of the enthalpies obtained at 25° are also included in Table I, since in a previous publication (Hinz and Jaenicke, 1973) only a graph was given. The enthalpies have been corrected for the heats of dilution in blank experiments.

Variation of Enthalpies of Dissociation with Temperature. The enthalpy values obtained under saturating conditions of the coenzyme are plotted as a function of temperature in Figure 2. The solid line represents the graph of a polynomial regression analysis of the first degree resulting in the equation for the temperature dependence of the dissociation enthalpy

$$-\Delta H = +1299T - 2294 \text{ cal/mol of LDH} \quad (1)$$

while the dashed curve is based on a polynomial regression analysis of the second degree, which gives the expression

$$-\Delta H' = 860T + 12T^2 + 928 \text{ cal/mol of LDH} \quad (2)$$

Standard errors of the estimates of ΔH and $\Delta H'$ are respectively 1796 cal/mol of LDH and 1683 cal/mol of LDH. The temperature T refers to degrees centigrade.

Variation of the Equilibrium Constant with Temperature. Table II shows dissociation constants as obtained by the calorimetric measurements and by equilibrium dialysis experiments at three temperatures. Equilibrium dialysis experiments have been performed on solutions identical with those used in the calorimetric enthalpy determinations (19 $\times 10^{-6}$ M LDH). Depending on temperature, equilibrium was reached within 5–8 hr and was 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).

According to fluorescence, optical absorption, and activity measurements, adsorption and denaturation phenomena did not occur during approach of equilibrium.

TABLE II: Temperature Dependence of the Dissociation Constant, K_D, of the NADH–LDH Complex as Determined by Thermal Titrations and Equilibrium Dialysis.[a]

Temp (°C)	K_D (μM)
10	6.3[b]
15	8.0[b]
25	8.6[b]
	7.4[c]
31.5	9.2[b]
	27.8[d]

[a] Equilibrium dialysis and calorimetric measurements at pH 7 in 0.2 M potassium phosphate buffer. [b] Dissociation constants calculated from the experimental ΔH values by a least-squares fitting procedure on the assumption of four independent and identical binding sites per LDH molecule, employing the enthalpy value under saturating concentrations of the coenzyme at the corresponding temperature. The value of 7.4 μM at 25° is lower than the value published (11.2 μM) (Hinz and Jaenicke, 1973) since after several additional experiments under saturating conditions of the coenzyme at 25° a ΔH value of 30 ± 1.5 kcal/mol of LDH seems to approximate the data better. [c] Dissociation constants derived from equilibrium dialysis measurements; enzyme concentration 19 μM. The values constitute averages of five determinations at each temperature.

Discussion

Thermal Titrations and Equilibrium Constants. Least-squares analysis of the calorimetric titration data on the assumption of four identical and independent binding sites per LDH molecule resulted in the equilibrium constants for 25 and 31.5° listed in Table II. While at 25° there is fairly good agreement between the values obtained by the two different methods, at 31.5° the dissociation constant arrived at by iterative fitting procedures applied to the calorimetric titration curve is larger by a factor of 3. This discrepancy has to be ascribed to increasing experimental uncertainty of the calorimetric measurements at that temperature due to increasing instability of the calorimeter. This instability manifests itself by experimental artifacts like sloping base lines and prolonged time required for temperature equilibration before the measurement. The somewhat poorer fit of the calorimetric titration curve at 31.5° to the theoretical binding curve is also expressed by the larger standard error of the ΔH estimate of ±2.1 kcal/mol of LDH compared with the ±1.4 kcal/mol of LDH at 25°.

Therefore the equilibrium dialysis measurements seem to be more reliable. They are in fairly good agreement with those derived from fluorescence titrations, taking the different experimental conditions into account (Stinson and Holbrook, 1973).

Temperature Variation of Thermodynamic Parameters for the Reaction of NADH with Pig Skeletal Muscle LDH. The strong dependence of the reaction enthalpy on temperature is illustrated in Figure 2. This phenomenon has been repeatedly observed in association reactions of biological macromolecules (Hearn *et al.*, 1971; Bode *et al.*, 1974) or the interaction of enzymes with coenzymes and inhibitors (Velick *et al.*, 1971; Hinz *et al.*, 1971). The calorimetric data on the temperature variation of ΔH are at variance with the indirect results derived by Stinson and Holbrook (1973) from the temperature dependence of the equilibrium constant. It is, in general, difficult to establish a set of equilibrium constants in the micromolar range with such an accuracy as to be able to decide in a van't Hoff plot whether a straight line or a curve approximates the experimental data best. Therefore it is not surprising that the van't Hoff plot usually fails to reveal any temperature dependence of the enthalpy. As a matter of fact with the information of a temperature dependent ΔH available one recognizes that a curve much better fits the experimental data given by Stinson and Holbrook in their Figure 7 for the muscle enzyme (Stinson and Holbrook, 1973).

As demonstrated in Figure 2, a polynomial regression analysis of the second degree approximates the enthalpy data slightly better than the linear fit. Although in many reactions known to exhibit strong temperature dependence of the binding enthalpy the ΔH apparently varies linearly with temperature, there is no law why that should be the case. As long, however, as experimental uncertainty does not allow an unambigous interpretation, the view that the binding reaction is characterized by a large decrease in apparent heat capacity might as well be adopted. Another model, which would equally well explain the temperature variation of the enthalpy, is based on the assumption of a temperature dependent equilibrium between two forms of the enzyme having different but temperature independent binding enthalpies. Presently accessible evidence, however, does not support this interpretation to apply to the LDH–NADH system.

Therefore the apparent thermodynamic parameters summarized in Table III are calculated on the basis of the Gibbs–Helmholtz equation using an enthalpy of 7500 cal/mol of NADH dissociated at 25° and assuming a temperature independent Δc_p of 325 cal/(deg mol of NADH). Whereas both enthalpy and entropy values show a marked variation with temperature the Gibbs free energy remains relatively constant, illustrating a compensatory effect of enthalpic and entropic factors.

For a discussion of molecular models to rationalize particularly the large Δc_p, the reader is referred to the literature (Tanford, 1968, 1970; Bode *et al.*, 1974; Sturtevant, 1972).

Comparison of Binding Enthalpies with Data from the Literature. Enthalpies for binding of NADH to pig and rabbit skeletal muscle LDH have been reported (Stinson and Holbrook, 1973: pig enzyme, $\Delta H = -31.5$ kcal/mol; 0.067 M sodium phosphate buffer (pH 7.2), fluorescence titrations; Czerlinski and Schreck, 1964: rabbit enzyme, $\Delta H = -32.4$ kcal/mol of LDH, phosphate buffer, ionic strength 0.01, total ionic strength 0.1 by addition of K_2SO_4 (pH 7), $T = 25°$; temperature jump experiments). In either case the ΔH values have been derived from the temperature dependence of the equilibrium constant. They are within the margin of experimental error of the techniques in agreement with the enthalpies obtained by calorimetry at 25°.

TABLE III: Apparent Thermodynamic Parameters for the Dissociation of NADH from Pig Skeletal Muscle Lactate Dehydrogenase at pH 7.0 in 0.2 M Potassium Phosphate Buffer.

Temp (°C)	ΔG^a (kcal/mol of NADH)	ΔH^b (kcal/mol of NADH)	ΔS^c (cal/mol of NADH deg)
5	6.73	1.05	−20.42
10	6.82	2.67	−14.66
15	6.88	4.30	−8.95
20	6.91	5.92	−3.38
25	6.91	7.55	+2.15
30	6.89	9.17	+7.52
35	6.84	10.79	+12.82
40	6.76	12.42	+18.07
45	6.66	14.04	+23.20
50	6.53	15.66	+28.25

[a] Calculated from the value of $\Delta G° = -RT \ln K_D$ obtained at 25° from the equilibrium dialysis measurements ($K_D = 8.6$ μM), using the Gibbs–Helmholtz equation and the enthalpies (per mole of binding site) given in column 3. [b] Calculated from the equation $\Delta H = 0.325T - 0.574$; T refers to °C. [c] $\Delta S = (\Delta H - \Delta G)/T$.

However, the large temperature dependence remained undetected because of the reasons mentioned before.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Miss Renate Schmidt and are indebted to F. X. Schmid for performing the equilibrium dialysis experiments. We express our appreciation to Mrs. R. Adiarte for her expert computer work.

References

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

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

Bode, W., Hinz, H.-J., Jaenicke, R., and Blume, A. (1974), *Biophys. Struct. Mechanism 1,* 55.

Czerlinski, G. H., and Schreck, G. (1964), *J. Biol. Chem. 239,* 913.

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

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

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

Jaenicke, R., and Knof, S. (1968). *Eur. J. Biochem. 4,* 157.

Mc Pherson, A., Jr. (1970), *J. Mol. Biol. 51,* 39.

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.

Sturtevant, J. M. (1972), *Methods Enzymol. 26C,* 227.

Tanford, C. (1968), *Advan. Protein Chem. 23,* 122.

Tanford, C. (1970), *Advan. Protein Chem. 24,* 1.

Velick, S. F., Bagott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10,* 779.