which the conformation change induced by AlATP and glucose has gone to completion prior to the addition of the originally limiting substrate. In Figure 1A, the time course of the conformation change in the presence of saturating glucose and limiting MgATP can be seen by the drop in the v_i/v_{ss} ratio to 1.0 by the time 20% of the MgATP has reacted. Only after 80% of the MgATP has reacted does the conformation change begin to reverse, and by the time the reaction has reached equilibrium, the replacement of AlATP by MgADP on the enzyme has completely reversed it. In these experiments, the high level of glucose present ensured that the sugar site was always occupied by glucose, and not by glucose 6-phosphate. In Figure 1B a similar phenomenon is seen, but because glucose was present initially at about half K_m levels, no more than $^1/_3$ of the enzyme had glucose on it at any time. The conformation change thus never went to completion, so that a burst was always seen when high glucose was added later. The kinetics seen in Figure 1 are thus fully consistent with the model we have postulated, and in particular show again the requirement for a sugar to allow slow binding behavior by AlATP.

Acknowledgments

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References

Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry 14*, 28.
Dunaway-Mariano, D., & Cleland, W. W. (1980) *Biochemistry 19*, 1506.
Frink, C. R., & Peech, M. (1963) *Inorg. Chem. 2*, 473.
Janson, C. A., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2572.
Kosow, D. P., & Rose, I. A. (1971) *J. Biol. Chem. 246*, 2618.
Morrison, J. F., & Cleland, W. W. (1980) *Biochemistry* (preceding paper in this issue).
Pattnik, R. K., & Pani, S. (1961) *J. Indian Chem. Soc. 38*, 379.
Peters, B. A., & Neet, K. E. (1976) *J. Biol. Chem. 251*, 7521.
Peters, B. A., & Neet, K. E. (1977) *J. Biol. Chem. 252*, 5345.
Schulze, I. T., & Colowick, S. P. (1969) *J. Biol. Chem. 244*, 2306.
Shannon, R. D. (1976) *Acta Crystallogr., Sect. A 32*, 751.
Shill, J. P., & Neet, K. E. (1971) *Biochem. J. 123*, 283.
Shill, J. P., & Neet, K. E. (1975) *J. Biol. Chem. 250*, 2259.
Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.
Womack, F. C., & Colowick, S. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5080.

Reactivity of Sulfhydryl Groups of the Flavoenzyme D-Lactate Dehydrogenase and Effect on Catalytic Activity†

Steven T. Olson‡ and Vincent Massey*

ABSTRACT: The zinc-dependent flavoenzyme D-lactate dehydrogenase from *Megasphaera elsdenii* possesses about nine sulfhydryl residues and no disulfide bonds per 55000 molecular weight subunit. Four to six of these sulfhydryl residues (depending on the pH) undergo a gradual oxidation with concomitant inactivation which can be prevented by dithiothreitol. A slow loss in the flavin binding ability of the enzyme occurs subsequent to sulfhydryl oxidation and thus precludes full reactivation by dithiothreitol. However, complete reactivation of enzyme retaining its flavin is obtained upon incubation with dithiothreitol. Both the oxidation and inactivation processes can be accelerated by catalytic levels of cupric ions. Approximately the same number of sulfhydryl residues are lost by copper inactivation (about five at pH 7.0) and dithiothreitol restores the starting activity per flavin. EDTA or pretreatment of buffers with Chelex 100 protects the enzyme from inactivation due to sulfhydryl oxidation even though up to two sulfhydryls are oxidized under these conditions. It is suggested that the spontaneous inactivation involves trace metal catalyzed oxidation of sulfhydryl residues. Three of the enzyme sulfhydryl residues react with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) at widely separated rates. The first reacts rapidly, and its modification is associated with a loss of ~95% of the catalytic activity. This thiol can be quantitatively modified by reaction with 1 equiv of DTNB or bromopyruvate, resulting in a 95% loss in catalytic activity. Enzyme samples allowed to spontaneously inactivate to varying degrees show a parallel loss of the first two thiols reactive with DTNB, suggesting that these residues are oxidized to a disulfide. Reaction of these two thiols with DTNB also results in the production of a disulfide enzyme since nearly 2 equiv of TNB is released when enzyme is reacted with 1 equiv of DTNB. *p*-(Chloromercuri)benzoate reacts with about seven of the enzyme thiols with complete inactivation occurring upon modification of four residues. Analysis of several enzyme preparations for their reactive thiol content indicates at most a 10% deviation from the expected value of 1 residue/flavin. Since the activity/flavin ratio has been found to vary up to 30% in several preparations, it is suggested that this cannot be accounted for by sulfhydryl oxidation and is likely to result from the demonstrated presence of modified flavins with differential catalytic activities.

A pyridine nucleotide independent D-lactate dehydrogenase catalyzes the first step in lactate oxidation in the anaerobic bacterium *Megasphaera elsdenii* (formerly known as *Peptostreptococcus elsdenii*) when grown in lactate (Baldwin & Milligan, 1964; Brockman, 1971). This enzyme, first identified by Baldwin & Milligan (1964), was later shown by Brockman & Wood (1975) to function as part of an electron transport chain involving two other enzymes, an electron-transferring flavoprotein (ETF)[1] and a butyryl-CoA dehydrogenase (BD-

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H). The three proteins, all flavin enzymes, served to couple the oxidation of D-lactate to the reduction of short-chain unsaturated acyl-CoAs via the following path of electron transfer:

D-lactate → D-lactate dehydrogenase → ETF → BDH → RCH=CHCOS—CoA

Brockman & Wood (1975) purified the D-lactate dehydrogenase to near homogeneity, but their resulting enzyme preparation was only partially reduced by excess D-lactate under anaerobic conditions (Brockman, 1971; Brockman & Wood, 1975). This anomaly was later resolved when we demonstrated that the Brockman & Wood procedure had removed most of the essential zinc cofactor (Olson & Massey, 1979). Homogeneous D-lactate dehydrogenase which had been reconstituted with zinc was rapidly and completely reduced by D-lactate anaerobically. Furthermore, the purified enzyme contained one atom of zinc and one FAD per 55 000 molecular weight subunit, the native enzyme being a dimer of apparently identical subunits (Olson & Massey, 1979).

Despite a full complement of zinc, homogeneous preparations of the enzyme exhibited varying activity/flavin ratios (AFR) between 100 and 140. This suggested that some inactive or partially active enzyme might still be present. A possible way of generation of inactive enzyme was indicated on finding a gradual inactivation of enzyme at 0 °C which could be prevented by DTT. Furthermore, rapid inactivation was observed to occur in the presence of substoichiometric cupric ions, known to catalyze sulfhydryl oxidation (Casola et al., 1966; Jocelyn, 1972).

The present paper reports on a more detailed study of the reactivity of the enzyme thiols, both toward metal-catalyzed oxidation and toward thiol reagents such as DTNB, bromopyruvate, and organic mercurials. The catalytic importance of a single reactive thiol residue is demonstrated.

Materials and Methods

Materials. D-Lactate dehydrogenase was prepared from cells of *M. elsdenii* according to Olson & Massey (1979). Enzyme concentrations were determined from the 454-nm absorbance by using ϵ_{454} = 13 000 M^{-1} cm^{-1} at 3 °C. Guanidine hydrochloride (ultrapure) was obtained from Schwarz/Mann and DTNB from Aldrich. The following chemicals were all obtained from Sigma: dithiothreitol, bromopyruvate (Na salt), NADH, *p*-(chloromercuri)benzoate, and rabbit muscle L-lactate dehydrogenase. Chelex 100 was from Bio-Rad, and Sephadex G-25 and Sephacryl S-200 were from Pharmacia. All other chemicals were reagent grade.

Methods. Assays of D-lactate dehydrogenase were done at 25 °C, pH 7.0, by using ferricyanide as an electron acceptor as described previously (Olson & Massey, 1979). The half-cystine content of the enzyme was determined by Dr. C. H. Williams, Jr., of this department, by oxidation to cysteic acid in dimethyl sulfoxide according to Spencer & Wold (1969). Absorption spectra were recorded with either a Cary 17 or a Cary 118 spectrophotometer.

Analyses of the total cysteine content of the enzyme were done in 4.2–4.8 M guanidine hydrochloride in 0.1 M potassium phosphate, pH 6.7–7.0, containing 50 mM EDTA. Typically, 1–5 μM enzyme (final concentration), freed of DTT by passage through a Sephadex G-25 column, was denatured in guanidine hydrochloride and the 412-nm absorbance recorded. At least a 20-fold excess of DTNB over enzyme was then added, and the 412-nm absorbance was monitored until an end point was reached. After subtraction of the DTNB absorbance, the ΔA_{412} was converted to moles of TNB released by using the determined extinction coefficient in guanidine hydrochloride, ϵ_{412} = 13 600 M^{-1} cm^{-1} (Riddles et al., 1979).

DTNB reactions with DTT-free native enzyme were done by preincubating 10–20 μM enzyme in 0.1 M potassium phosphate buffer at a suitable temperature and pH and recording the 412-nm absorbance. An aliquot of a 10 mM stock DTNB solution was added and, after mixing, the increase in A_{412} was followed with time. Since an end point could not be obtained (see Results), the data were analyzed by the Guggenheim method (Guggenheim, 1926). The validity of this procedure for parallel first-order reactions was verified mathematically and tested by simulation. The procedure involves plotting the log of the absorbance change from any time, t, over a suitable constant time interval, Δt, vs. t. Δt should be several times greater than $t_{1/2}$ (Guggenheim, 1926) in order to obtain accurate rate constants. At pH 7.0, 20 °C, the DTNB reaction of D-lactate dehydrogenase yields three phases, the first of which is too fast to be measured by conventional spectrophotometry. A Δt of 10 min was used to obtain the rate and amplitude of the second phase ($t_{1/2} \sim 3$ min). Since this Δt is too small for a reliable estimate of the rate of the third phase, a second plot using a Δt of 100 min was made to ensure an accurate determination of the third phase ($t_{1/2}$ = 50–60 min). This slowest phase could then be subtracted to yield a biphasic curve. A further subtraction of the slower of the remaining two phases gave the amplitude of the fastest phase. The biphasic curves obtained in the DTNB reaction of pH 6.3, 3 °C, were analyzed in like manner. Rate constants were obtained from the slope of each resolved linear phase.

The total absorbance change associated with each of the phases, as determined in the last paragraph, can be calculated from the intercept of each phase and the rate constant associated with the particular phase. (This is necessary because the intercept represents the absorbance change associated with the first Δt, 5 or 10 min, used in the Guggenheim analysis.) The recently reported ϵ_{412} = 14 200 M^{-1} cm^{-1} (Riddles et al., 1979), obtained with scrupulously purified TNB, was used to convert ΔA_{412} to moles of thiol reacted. Titrations of the enzyme with DTNB were performed at 0 °C. Varying amounts of a dilute DTNB solution up to and beyond 1 equiv were added to several identical aliquots of enzyme (free of DTT). Since preliminary experiments had shown that several hours were required for complete release of TNB at this temperature, the reactions were allowed to run overnight on ice and then were analyzed for activity and TNB released the next day. Correction was made for small activity losses (up to 10%) occurring in the control sample.

Titrations with bromopyruvate and PCMB were conducted similarly to the DTNB titrations. Additions from stock reagent solutions were made to several identical samples of enzyme and allowed to react overnight. This was shown to be sufficient time for completion of the reaction. With the exception of the absorbance titration with PCMB (Figure 6, right panel), purified enzyme having an FAD content of 0.96 equiv/55 000 molecular weight subunit was used for all studies. Data obtained from the PCMB titration of Figure 6, right panel, were corrected for the presence of an estimated 15% apoenzyme in this particular preparation. Bromopyruvate concentrations were determined by using rabbit muscle L-lactate de-

[1] Abbreviations used: ETF, electron-transferring flavoprotein; BDH, butyryl-CoA dehydrogenase; AFR, activity/flavin ratio; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate anion; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol.

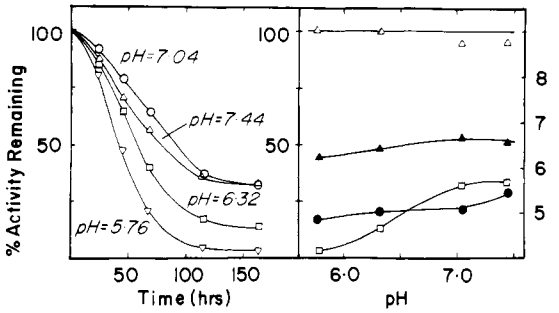


FIGURE 1: Inactivation of D-lactate dehydrogenase at 0 °C at different pH values and correlation with total sulfhydryl content. Separate samples of 35 μM enzyme (freed of DTT by passage through Sephadex G-25) of AFR = 106 in 0.1 M potassium phosphate buffers of varying pH were incubated at 0 °C and their activity was monitored with time (left panel). (Right panel) Total cysteine content of enzyme samples from the experiments of the left panel determined on 0.1-mL samples in 4.2 M guanidine hydrochloride with DTNB (see Methods) after 3 days (▲) and again after 7 days (●). The residual enzyme activity after 7 days is shown for the samples in the absence of DTT (□) and in control experiments incubated for 7 days in the presence of 1.7 mM DTT (△).

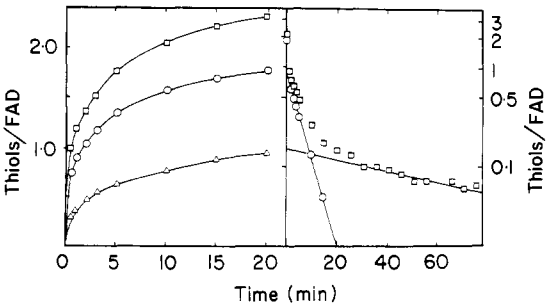


FIGURE 2: Reaction of D-lactate dehydrogenase with DTNB at pH 7.0. (Left panel) The reaction of 7.0 μM enzyme in 0.1 M potassium phosphate, pH 7.0, 20 °C, with 100 μM DTNB. Absorbance values at 412 nm have been converted to thiols reacted per flavin by using ϵ_{412} = 14 200 M^{-1} cm^{-1} for TNB. (□) Starting enzyme, AFR = 113; (○) enzyme allowed to inactivate at 0 °C to an AFR value of 73; (△) enzyme of AFR = 38. (Right panel) Analysis of the DTNB reaction with AFR = 113 enzyme by the method of Guggenheim (see Methods). (□) Plot of log $[A(t + \Delta t) - A(t)]$ vs. t, where A denotes absorbance at 412 nm and t the time. A time interval, Δt, of 10 min was used for this plot. (○) After subtraction of the slow phase of the primary plot.

hydrogenase and NADH. Concentrations of PCMB stock solutions were determined from the absorbance at 233 nm by using ϵ_{233} = 17 000 M^{-1} cm^{-1} (Boyer, 1954).

Results

Sulfhydryl Content of D-*Lactate Dehydrogenase.* Analysis of homogeneous D-lactate dehydrogenase for total sulfhydryls after oxidation to cysteic acid (Spencer & Wold, 1969) gave an average of 8.6 mol of half-cystine per mol of protein subunit. Determination of the total cysteine content by reaction with DTNB in 4.2–4.8 M guanidine hydrochloride yielded an average value of 8.9 residues/subunit (range 8.7–9.2). The enzyme therefore contains no disulfide bonds.

Loss of Enzymic Activity upon Removal of DTT. DTT was routinely present during purification of D-lactate dehydrogenase since it has been reported to prevent activity losses (Brockman, 1971; Brockman & Wood, 1975). We indeed found that removal of DTT from the purified enzyme caused a spontaneous inactivation of the enzyme, which did not occur when DTT was present (Olson & Massey, 1979). Figure 1 shows the time-dependent inactivation that occurs at 0 °C at several pH values. Extensive inactivation occurs at all pH values studied but requires several days. The rate of inactivation is slowest at pH 7.0, increasing both above and below this pH. A much more rapid inactivation was found at higher pH. In one experiment at pH 8.5 in 0.1 M pyrophosphate buffer at 0 °C, half of the enzyme activity was lost in an hour and nearly complete inactivation was reached within 10 h. While this pattern of relative reaction rates was consistently observed, there was considerable variation in the actual inactivation rates when different buffers or even different lots of the same buffer were used. These results suggest that the variability may be due, in part at least, to trace metal contamination. This is dealt with more fully in a later section.

The right panel of Figure 1 shows that complete protection is rendered by DTT against activity loss over the 7-day course of the experiment. This suggested that an oxidation of cysteine residues might be the cause of the inactivation process. Total thiol analysis in 4.2 M guanidine hydrochloride (right panel, Figure 1) confirmed that significant loss of DTNB-reacting thiols was occurring concomitant with the inactivation. The nearly complete inactivation observed at pH 5.8 over 7 days was associated with a drop in total thiols from 8.8 to 4.8 per flavin, equivalent to 4.0 cysteine residues. The number of thiols lost after complete inactivation at higher pH values, estimated by extrapolation of the results in Figure 1, was significantly greater. At pH 7.4, the extrapolated loss of sulfhydryl groups increased to 5.3/flavin. In another experiment at pH 8.7, enzyme was allowed to inactivate spontaneously for 1 day, which resulted in 99% inactivation. Determination of the total thiol content of the inactivated enzyme showed that 5.7 thiols/flavin was lost.

Consistent with the inactivation process involving the oxidation of cysteine residues, it was found that the inactivated enzyme could be reactivated by DTT. Although the extent of reactivation was found to decrease with prolonged incubation, this was shown to be the result of a slow loss of flavin binding ability. In one typical experiment, enzyme (AFR = 122) that had been inactivated to an AFR of 10 at pH 6.3 over 4 days was reactivated to AFR = 77 after 2 days of incubation with 2 mM DTT. When passed through Sephadex G-25, the AFR increased to 94 and the A_{274}/A_{454} ratio was significantly higher (6.9), indicating that ~20% of the flavin had been lost. Further incubation with 2 mM DTT for another day brought the AFR up to 110. In several other experiments performed, it was found that nearly complete recovery of the starting AFR could be obtained by incubation with 1 to 2 mM DTT (after removing unbound flavin by passage through Sephadex G-25).

Differential Reactivity of Cysteine Residues with DTNB at pH 7.0. As a means of differentiating individual cysteine residues of the enzyme, the reaction of the native enzyme with DTNB was investigated. Figure 2 shows the results obtained when enzyme is reacted at pH 7.0, 20 °C, with a large molar excess of DTNB (pseudo-first-order conditions). TNB release equivalent to 1 thiol/flavin occurs very rapidly (~0.5 min) while a second thiol is reacted by 10 min. Slow continued release of TNB takes place with three thiols reacting within ~2 h. No end point was obtained for this reaction as a much slower but significant rate of TNB formation beyond three thiols took place. This accounted for the reaction of an additional thiol in the next 20 h and even further reaction beyond this time. (DTNB hydrolysis was negligible.) Since the enzyme was known to be susceptible to slow thermal denaturation beyond 2 h at this temperature, this very slow reaction is probably due to exposure of buried sulfhydryls accompanying denaturation.

When the activity of the enzyme was monitored during the reaction, nearly all of the activity was lost (94%) after the

Table I: Correlation of Loss of Activity with Loss of Enzyme Thiols[a]

AFR	thiols/E·FAD (phase 1)	thiols/E·FAD (phase 2)
113	0.94	1.05
73	0.68	0.72
38	0.27	0.35

[a] The enzyme thiols reacting with DTNB in the very rapid (phase 1) and rapid (phase 2) phases were analyzed as shown in Figure 2.

reaction of the first thiol was complete. A slow continued loss of the residual activity occurred with further thiol modification.

A resolution of the several kinetic phases obvious from Figure 2 could be obtained by analyzing the data according to the Guggenheim method (Guggenheim, 1926) which does not require an end point (right panel, Figure 2; see Methods for a description of this analysis). Subtraction of the slowest phase gave a linear phase corresponding to a first-order process with a $t_{1/2}$ of 3.1 min. This phase should correspond to the second thiol reacting with DTNB. Extrapolation to the zero-time axis gives the number of thiol residues reacted in this phase in the first 10 min of reaction. (A time interval of 10 min was used for this analysis; see Methods.) Knowledge of the observed rate constant then allows correction of this value so that the total number of thiols corresponding to this phase can be determined. A value of 1.05 thiols/FAD was obtained. An initial rapid phase responsible for activity loss is evident, corresponding to 0.94 thiol/FAD. The slowest phase ($t_{1/2} \sim 60$ min) represented 1.2 thiols/FAD. Thus, three distinct sulfhydryl residues could be distinguished on the basis of their DTNB reactivity.

It was of interest to see whether any of these DTNB-reactive cysteines were being oxidized during the spontaneous inactivation of the enzyme. Figure 2 shows the DTNB reaction of enzyme allowed to inactivate at pH 7.0 to AFR values of 73 and 38. The two cysteines reacting in phases 1 and 2 of the DTNB reaction were lost concomitant with inactivation. Three distinct phases were again resolved with similar rates in each case (Figure 2, right panel). Table I gives the number of thiols associated with phases 1 and 2. These two thiols appear to be lost to the same extent and in proportion to the activity loss. Substantially smaller losses were observed for the slowly reacting third thiol. Only 0.06 thiol/FAD was lost in the third phase in enzyme of AFR = 73; the loss increased to 0.34 thiol/FAD in AFR = 38 enzyme.

Reaction with DTNB at pH 6.3. The reaction was done at a lower pH and temperature in order to further quantitate the reaction of the very reactive cysteine residue with DTNB. The reaction of AFR = 122 enzyme with DTNB under pseudo-first-order conditions (~10-fold excess of DTNB) at pH 6.3 is shown in the left panel of Figure 3. The reaction of the first thiol is significantly slowed under these conditions. Again, since no end point could be obtained, the data were analyzed by the Guggenheim method. A biphasic reaction was obtained (Figure 3, right panel). After subtracting the slow phase, a linear phase ($t_{1/2}$ = 1.2 min) corresponding to the reaction of 0.90 thiol/FAD resulted. As expected for a second-order reaction, the observed rate constant varied linearly with DTNB concentration (data not shown).

Also shown in Figure 3 (left panel) is the same reaction performed on enzyme inactivated to varying extents at pH 6.3. In confirmation of the results obtained at pH 7.0, the spontaneous inactivation at this pH was accompanied by the loss of the very reactive cysteine. The inset in Figure 3 shows a plot of AFR vs. the fraction of this cysteine remaining. A

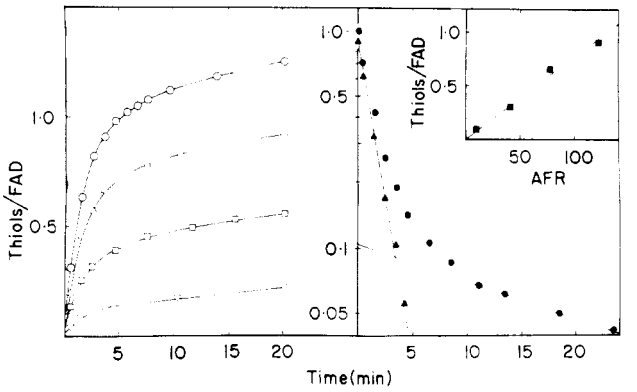
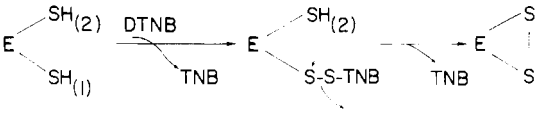


FIGURE 3: Reaction of D-lactate dehydrogenase with DTNB at pH 6.3. (Left panel) 9.5 μM enzyme in 0.1 M potassium phosphate, pH 6.3, 3 °C, was reacted with 170 μM DTNB. (○) Data obtained for AFR = 122 enzyme (converted to thiols per FAD by using ϵ_{412} = 14 200 M^{-1} cm^{-1}). The reaction was then repeated (identical conditions) after inactivation of the enzyme at 3 °C to AFR values of 78 (△), 41 (□), and 9.6 (▽). (Right panel) Guggenheim analysis of data shown in the left panel for the DTNB reaction with AFR = 122 enzyme. The primary plot of the log of the 412-nm absorbance change over 5-min time intervals vs. time is shown by the solid circles. The solid triangles were obtained after subtracting the slow phase. The inset shows a plot of enzyme AFR vs. thiols per FAD in the rapid phase (obtained from Guggenheim plots of the data shown in the left panel).

linear correlation is observed which extrapolates to an AFR of 131 at 1 thiol/flavin.

Reaction with 1 equiv of DTNB. The disappearance of two DTNB-reactive cysteine residues accompanies the spontaneous inactivation of D-lactate dehydrogenase. It seemed likely that such a stoichiometric loss of two thiols was the result of oxidation to a disulfide bond. If these two cysteines are favorably situated for disulfide formation, the second equivalent of TNB released in the DTNB reaction might arise from a thiol–disulfide interchange of the phase 2 thiol with the mixed disulfide of the phase 1 thiol and TNB, according to the scheme



Such a scheme could be tested by reacting the enzyme with 1 equiv of DTNB. If disulfide formation can occur, 2 equiv of TNB should be released. Furthermore, since activity loss is associated with modification of the first reacting thiol, the reactive cysteine residue should be titratable with DTNB.

The titration of AFR = 91 enzyme is shown in Figure 4. The release of 1.84 equiv of TNB per equiv of DTNB added occurs up to the activity end point, demonstrating the formation of disulfide concomitant with activity loss.[2] Also in agreement with the above scheme, the observed rate of the second phase of the DTNB reaction at pH 7.0 (Figure 2) was largely independent of DTNB concentration, as expected for an intramolecular process. Half-times of 3.5–2.9 min were found for this phase when the DTNB concentration was raised from 50 to 300 μM (7.2 μM enzyme). The possibility that intermolecular disulfide formation was occurring could be ruled

[2] The fact that slightly less than 2.0 equiv of TNB is released per equiv of DTNB is probably due to a change in the A_{412} value of the enzyme flavin as a result of the reaction. This is suggested by the fact that when the DTNB-reacted enzyme was subjected to Sephadex G-25 gel filtration, incomplete recovery of the expected flavin absorbance was obtained, even though no loss of FAD could be detected by analysis of later fractions.

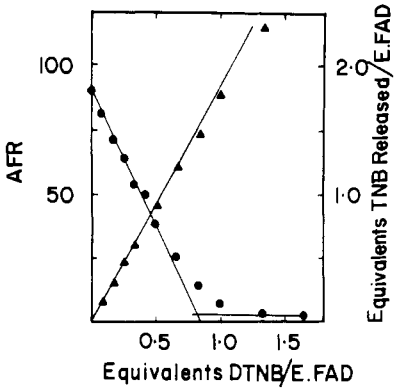


FIGURE 4: DTNB titration of D-lactate dehydrogenase. To several 0.2-mL samples of 31 μM enzyme in 0.1 M potassium phosphate, pH 7.0, plus 0.3 mM EDTA were added increasing aliquots of a 104 or 1040 μM DTNB solution in the same buffer, and the samples were incubated at 0 °C overnight. Residual activity was then determined and corrected for both dilution and an 8% loss in activity of the control sample (●). All samples were then diluted to 0.8-mL final volume and the 412-nm absorbance was determined. The absorbance data were then converted to equivalents of TNB released per equivalent of DTNB (after correction for dilution) (▲). The concentration of DTNB solutions was determined by addition of excess DTT to a diluted aliquot.

out since disulfide enzyme eluted at the same volume (704 mL) as native enzyme (697 mL) when passed through a 1200 mL Sephacryl S-200 column.[3]

A linear decrease in activity occurs as increasing substoichiometric DTNB is added, which is consistent with a stoichiometric reaction of DTNB with a very reactive cysteine residue. The small residual activity is slowly lost with excess DTNB. The end point occurs after 0.81 equiv of DTNB has been added, which would correspond to an AFR of 112 for fully active enzyme. However, the original AFR of this enzyme preparation before DTT removal was 120. In the interval between DTT removal and the DTNB titration, the AFR value had declined to 91. The DTNB titration of the reactive thiol thus indicates that the loss of activity occurring after removal of DTT can be fully accounted for by oxidation of this thiol residue, which implies that the initial AFR of 120 corresponds to 1.0 equiv of this reactive thiol. As enzyme preparations with AFR values as high as 140 have been obtained not infrequently, it is clear that other factors, in addition to the oxidation state of the reactive thiol, must contribute to the AFR value. This point will be considered in more detail under Discussion.

Formation of the disulfide enzyme is reversible. Upon overnight incubation with 1 to 2 mM DTT, full reactivation is achieved. On storage, however, the disulfide enzyme slowly loses flavin irreversibly.

Reaction with Bromopyruvate. Bromopyruvate has been shown to react with the sulfhydryl groups of several enzymes for which pyruvate is a substrate or product (Gale, 1961; Fonda, 1976; Yun & Suelter, 1979; Cybulsky et al., 1979; Mulet & Lederer, 1977). Rapid inactivation resulted when small molar excesses of the reagent were added to D-lactate dehydrogenase. Further study revealed that only 1 equiv was necessary to abolish ~95% of the activity. Figure 5 shows a titration of the enzyme (AFR = 107) with this reagent. As with DTNB, graded substoichiometric additions brought about

[3] It should be pointed out that since D-lactate dehydrogenase is a dimer of identical subunits (Olson & Massey, 1979), an intramolecular disulfide could include either an intrasubunit or an intersubunit disulfide. In the case of the latter situation, nonidentical thiols must be involved in order to fit our kinetic data.

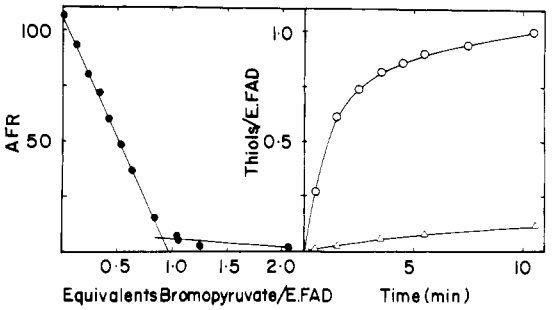


FIGURE 5: Titration of the reactive cysteine of D-lactate dehydrogenase by bromopyruvate. (Left panel) To several 0.10-mL samples of 19 μM enzyme in 0.1 M potassium phosphate, pH 7.0, were added increasing aliquots of a 38.8 or 388 μM bromopyruvate solution in the same buffer. After overnight reaction at 0 °C, samples were assayed for residual activity and these values were then corrected for both dilution and a 13% loss in activity of the control sample. The data are plotted as AFR vs. equivalents of bromopyruvate added. (Right panel) A separate sample of 19 μM enzyme in 0.1 M potassium phosphate, pH 7.0, was reacted with 1.0 equiv of bromopyruvate overnight at 0 °C, resulting in inactivation to AFR = 5. A sample was then diluted with 0.1 M potassium phosphate, pH 6.3, plus 0.3 mM EDTA to 3.5 μM enzyme and then reacted with 100 μM DTNB (△). An untreated control sample diluted to the same final enzyme concentration was reacted with 100 μM DTNB (○).

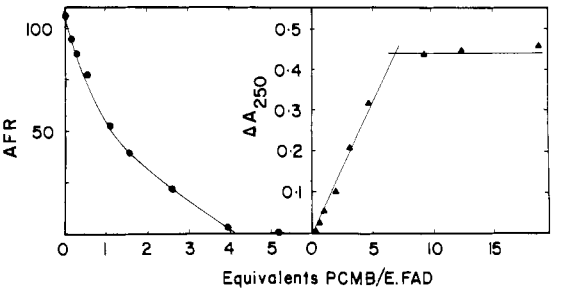


FIGURE 6: (Left panel) To several 0.1-mL samples of 9.2 μM enzyme (AFR = 107) in 0.1 M potassium phosphate, pH 7.0, were added increasing aliquots of a 23.8 or 238 μM PCMB solution in the same buffer, and the samples were incubated overnight at 0 °C. The residual activity was then determined and corrected for dilution and plotted as AFR vs. equivalents of PCMB added. (Right panel) To several 0.9-mL samples of 8.5 μM enzyme (AFR = 118) were added increasing aliquots of a 238 μM PCMB solution. After overnight incubation at 0 °C, the 250-nm absorbance of each sample was read and corrected for dilution. After subtraction of absorbance due to PCMB, the ΔA_{250} was plotted vs. equivalents of PCMB added.

a linear decrease in activity. Again, complete inactivation was not achieved with 1 equiv; further additions of inhibitor caused a gradual decrease in the residual activity. A stoichiometric reaction of a reactive cysteine residue is indicated by these data.

The right panel of Figure 5 demonstrates that the reactive cysteine of the enzyme has indeed disappeared after reaction with 1 equiv of bromopyruvate. Subsequent reaction with excess DTNB at a pH of ~6.3 shows that rapidly reacting cysteine has been lost (compare with untreated enzyme). The end point of the bromopyruvate titration occurs at 0.91 equiv, corresponding to an extrapolated AFR of 110 for enzyme possessing a full complement of its reactive cysteine. Since the preparation of enzyme used for this experiment was the same as that used for the DTNB titration, but at an earlier time after removal of DTT, the small loss in the reactive thiol can again be accounted for by oxidation subsequent to DTT removal.

Titration with PCMB. The tight binding of mercurials to sulfhydryl groups suggested that this reagent might also react in a stoichiometric fashion with the enzyme thiols. Upon titration of the enzyme with *p*-(chloromercuri)benzoate

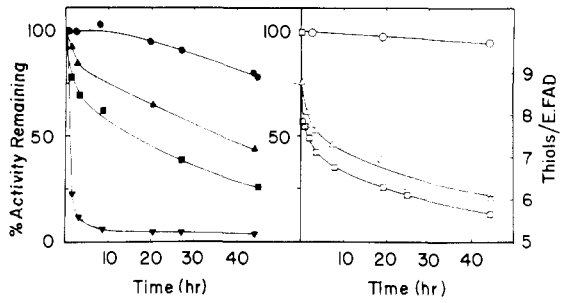


FIGURE 7: (Left panel) To several 0.20-mL samples of 30 μM enzyme (AFR = 110) in 0.1 M potassium phosphate, pH 7.0, were added increasing aliquots of a 100 μM $CuSO_4$ solution. Activities of the samples were monitored with time and corrected for dilution. Results are presented for final Cu^{2+}/flavin ratios of 0 (●), 0.08 (▲), 0.17 (■), and 0.50 (▼). (Right panel) To 2.8 mL of 24 μM enzyme (AFR = 112) in 0.1 M potassium phosphate, pH 7.0, was added 20 μL of 1 mM $CuSO_4$, giving a Cu^{2+}/FAD ratio of 0.3. The sample was incubated at 0 °C, and at varying intervals the activity and total thiols were determined as described under Methods. Curve (□) shows the activity losses with time, and curve (Δ) gives the residual thiols per FAD with time. Curve (○) shows the activity in an untreated enzyme control.

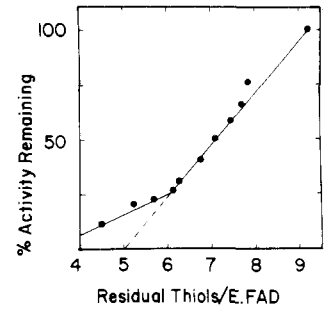


FIGURE 8: Data taken from the experiment shown in the right panel of Figure 7 plotted as thiols per FAD vs. residual activity.

(PCMB), the absorbance at 250 nm which monitors the formation of bound mercurial (Boyer, 1954), increases in a roughly linear fashion to an end point (Figure 6). On the assumption that a stoichiometric reaction is occurring, the end point indicates that about seven thiols have reacted. This number agrees well with the value, 6.8, obtained from the total ΔA_{250} and the published extinction change of $\Delta\epsilon_{250}$ = 7600 M^{-1} cm^{-1} for formation of the mercurial mercaptide chromophore (Boyer, 1954). When the effect on the enzyme activity is examined (Figure 6, left panel), a gradual decrease in activity takes place without any clear separation into phases; complete inactivation is reached after the addition of ~4 equiv of mercurial. This reagent, therefore, does not discriminate between the enzyme thiols as well as bromopyruvate or DTNB. Activity could be completely restored with 1 mM DTT (overnight incubation) to enzyme reacted with PCMB up to 2 equiv. Progressively poorer reactivation occurred with enzyme reacted with greater amounts. This might be the result of flavin loss.

Inactivation by Cu^{2+}. The oxidation of protein sulfhydryl groups by trace metals (especially Cu^{2+}) has been well documented for several enzymes (Casola et al., 1966; Boothe & Folk, 1969). Since buffers are known to contain trace metal contaminants, this appeared to be the most logical explanation for the observed spontaneous inactivation. The effect of Cu^{2+} on the enzyme was therefore investigated.

Copper was found to be a potent inhibitor of D-lactate dehydrogenase (Olson & Massey, 1979). Figure 7 (left panel) shows the effect of substoichiometric levels of Cu^{2+} on the catalytic activity. Complete inactivation requires only catalytic quantities and appears to occur in two distinct phases: a relatively rapid decline, the extent of which increases with increasing Cu^{2+}, followed by a slower rate of inactivation. Even at higher levels of Cu^{2+}, the inactivation levels out at a residual activity of ~5% which is lost extremely slowly.[4]

[4] Inactivation by levels of Cu^{2+} in excess of the enzyme concentration was found to be partially reversible by EDTA. This suggested a secondary source of enzyme inhibition as involving simple metal binding not accompanied by sulfhydryl oxidation at these Cu^{2+} concentrations. Such inhibition by excess metal has also been observed in the presence of an excess of the redox inactive metal Zn^{2+}. No EDTA reversibility was found at the substoichiometric levels of Cu^{2+} used in Figures 7 and 8, suggesting that the observed inactivation was entirely due to sulfhydryl oxidation.

Table II: Effect of EDTA and DTT on the Thiol Titer of D-Lactate Dehydrogenase[a]

	after 3 days		after 7 days	
	% act. remaining	thiols/ E·FAD	% act. remaining	thiols/ E·FAD
no additions	40	6.4	13	5.0
+DTT	103	8.8	101	
+EDTA	66	8.7	37	7.2

[a] Enzyme was incubated at pH 7.0, 0 °C, as in Figure 1. When DTT was present, it was at a concentration of 1.7 mM; when EDTA was added, it was at a concentration of 0.33 mM.

Figure 7 (right panel) shows the inactivation of enzyme by 0.3 equiv of Cu^{2+} per flavin and the thiol analyses on samples during the inactivation. The rate of inactivation is nicely paralleled by a loss in thiol residues. From these data, the number of thiols per flavin was plotted vs. the residual activity (Figure 8) to determine the number of thiols lost upon complete inactivation. A linear correlation is followed down to ~25% residual activity which extrapolates to a loss of ~4 thiols/FAD. Complete inactivation is associated with the loss of additional thiols.

Enzyme inactivated by Cu^{2+} can be reactivated by incubation with 1–2 mM DTT for several days. As was found with the spontaneous inactivation, a slow loss of flavin precludes quantitative reactivation, but complete return of the starting AFR is typically obtained when the reactivated enzyme is dialyzed to remove any FAD which is no longer tightly bound.

The obvious similarity of the copper inactivation to the spontaneous inactivation seemed to favor the hypothesis that traces of Cu^{2+} or some other metal ion were responsible for this latter process. To further test this idea, enzyme was incubated at 0 °C without DTT in the presence of EDTA, which would be expected to chelate any free metals. Table II compares the extent of inactivation and thiol oxidation occurring in the presence and absence of EDTA. Although inactivation does take place in the presence of EDTA, it is significantly slower than in its absence. Furthermore, substantially fewer thiols are lost when EDTA is present. In fact, the observed inactivation in the presence of EDTA could be shown to be due completely to the removal of zinc. This was initially suggested when no reactivation of the EDTA-inhibited enzyme was obtained by incubation with DTT. After removing the EDTA by gel filtration, it was found that both DTT and zinc were required to completely reactivate the inhibited enzyme.

Thus, EDTA inhibits thiol oxidation associated with enzyme inactivation. But it is clear that even in the presence of EDTA, some thiol oxidation occurs which is not associated with enzyme inactivation. The same phenomenon could be observed if the spontaneous inactivation was carried out in buffer pretreated with Chelex 100 to remove trace metals. The

control experiment of Figure 7 (right panel) was performed in Chelex-treated buffer. While only 6% of the activity was lost, the total thiols decreased from 9.2 to 7.5 per enzyme flavin. It therefore appears that 1 to 2 thiols can be oxidized without metal catalysis. Furthermore, their loss appears to have a negligible effect on the enzymic activity.

Discussion

A study of the sulfhydryl groups of D-lactate dehydrogenase was undertaken in order to determine whether an irreversible oxidation of labile sulfhydryl groups could account for the variable AFR values exhibited by different preparations of the enzyme. The results presented in this paper clearly show that out of approximately nine total sulfhydryl groups per enzyme subunit, four to six (depending on the pH) undergo a slow spontaneous oxidation with concomitant inactivation. That enzyme inactivation is the result of sulfhydryl oxidation was implied from the ability of DTT to both protect the enzyme from inactivation or accompanying sulfhydryl oxidation and to restore the activity to inactivated enzyme.

Several pieces of evidence indicate that the spontaneous oxidation of sulfhydryl groups of D-lactate dehydrogenase is largely catalyzed by trace metal buffer contaminants. First, cupric ions accelerate both the inactivation and oxidation of sulfhydryl groups in a catalytic fashion. The copper inactivation was indistinguishable from the spontaneous inactivation in that (a) activity loss paralleled a loss of sulfhydryl groups, (b) a similar number of sulfhydryl groups were lost upon complete inactivation, and (c) DTT reactivated the inactivated enzyme. Second, EDTA addition or pretreatment of buffers with Chelex 100 severely retarded sulfhydryl oxidation. The inactivation of several enzymes has likewise been demonstrated to occur via a metal-catalyzed oxidation of thiol residues (Casola et al., 1966; Boothe & Folk, 1969; Glazer & Smith, 1971; Thorpe & Williams, 1975). It was rather surprising to find that removal of trace metals by the latter treatments did not completely abolish sulfhydryl oxidation. Up to two sulfhydryl groups could be oxidized with little effect on the activity. A corollary is that those sulfhydryl residues important for enzyme activity are oxidized in a metal-catalyzed process.

A study of the reactivity of the sulfhydryl groups of D-lactate dehydrogenase with three sulfhydryl reagents served to identify specific thiols lost during the spontaneous inactivation, including those important for catalytic activity. Three cysteines of the enzyme were found to react with DTNB at widely separated rates. One was very reactive, and its modification was shown to be associated with loss of ~95% of the enzyme activity. Modification of the second cysteine was shown to occur via intramolecular displacement of TNB from the very reactive residue to give an intramolecular protein disulfide. This was indicated by both the independence on DTNB concentration of the observed first-order reaction rate for modification of this residue and the release of nearly 2 equiv of TNB when enzyme was reacted with only 1 equiv of DTNB. It logically follows that these two cysteines are proximally located in the tertiary structure of the enzyme. The identification of neighboring cysteines in several other enzymes has similarly been deduced by demonstrating the formation of an intramolecular disulfide upon reaction with DTNB (Connellan & Folk, 1969; Okabe et al., 1970; Flashner et al., 1972; Cybulsky et al., 1979).

Of the three DTNB-reactive cysteines, it was found that the two shown to form a disulfide with DTNB were lost during spontaneous inactivation in parallel and concomitant with inactivation. These data strongly suggest that the spontaneous inactivation process involves the oxidation of these two DTNB-reactive cysteines to a disulfide and accounts for virtually all the observed inactivation associated with sulfhydryl oxidation.

The extent to which DTT can reverse sulfhydryl oxidation is a crucial point in deciding the importance of autoxidation in producing variable AFR values. An earlier report presented data showing that the copper-inhibited enzyme gave progressively poorer reactivation when allowed to age (Olson & Massey, 1979). It was confirmed in the present study that enzyme oxidized spontaneously or with added copper gave poorer reactivation the longer oxidation was allowed to proceed. However, full restoration of the starting AFR was obtained with DTT in all cases if the oxidized enzyme was first passed through a gel filtration column, indicating that flavin loss was responsible for the inability to restore the starting activity. Thus, in terms of flavin binding ability, as expressed by the AFR value, the oxidation/inactivation process is fully reversible. The use of DTT in the enzyme preparation would thus be expected to reverse any oxidation which may have occurred and maintain the enzyme's sulfhydryl groups in a reduced state. This would argue against sulfhydryl oxidation as contributing to the AFR variability in the presence of DTT.

Further evidence indicating that spontaneous sulfhydryl oxidation is not related to AFR variability of DTT-protected enzyme comes from correlations of AFR with oxidation of the reactive cysteine shown to be important for catalytic function. A kinetic analysis of the rapid phase of the DTNB reaction at pH 7.0 with AFR = 113 enzyme indicated that the enzyme possessed 0.94 mol of reactive cysteine per mol of flavin. A similar kinetic analysis of data obtained at pH 6.3 for this same reaction but with a different enzyme preparation (AFR = 122) gave a value of 0.90 reactive thiol residues/FAD. Unfortunately, the magnitude of error associated with these data (5–10%) precludes the conclusion that these numbers are different from the expected theoretical value of 1.0. An independent quantitation of this reactive sulfhydryl group could be obtained by activity titration with either DTNB or bromopyruvate. The end points of these titrations indicated reactive thiol stoichiometries of 0.81/flavin for AFR = 91 enzyme (DTNB titration) and 0.91/flavin for AFR = 107 enzyme (bromopyruvate titration). However, since the original AFR of the enzyme used for these experiments was 122 (identical with the preparation used for the pH 6.3 DTNB reaction of Figure 3), the reactive thiol losses indicated from these titrations can be completely accounted for by oxidation occurring subsequent to removal of DTT from the enzyme. These and similar experiments with enzyme preparations possessing variable AFR values have uniformly shown the content of the reactive thiol to fall within the range 0.9–0.96/E·FAD. On the contrary, the observed AFR variability ranges between 100–140, close to a 30% variation. The sum of this evidence indicates that sulfhydryl oxidation cannot account for the observed variability of DTT-protected enzyme.

Another possible source of AFR variation does exist. Studies of the flavin adduct formed during inhibition of D-lactate dehydrogenase by the suicide inhibitor 2-hydroxy-3-butynoate demonstrated that native enzyme contains up to 10% modified flavins, but the actual content was found to vary from preparation to preparation (Olson et al., 1979; Olson, 1979). If these modified flavins were active, possessing different AFR values, variation of the AFR would be expected. Several observations made with this enzyme lend support to this hypothesis. First, apoenzyme reconstituted with purified FAD has an AFR of ~110. Second, several artificial enzymes have been prepared by replacing the native coenzyme with struc-

turally modified flavins (modification being restricted to the isoalloxazine moiety of FAD) which indeed possess a higher AFR than the native enzyme (S. T. Olson and V. Massey, unpublished observations). Thus, enzyme reconstituted with 8-α-hydroxy-FAD had an AFR value of 166 and enzyme reconstituted with iso-FAD had an AFR of 126. While the modified natural enzymes do not contain either of these flavins, the possibility is clear that the variable AFR values of different enzyme preparations may reflect the content of flavins of unknown structure.

References

Baldwin, R. L., & Milligan, L. P. (1964) *Biochim. Biophys. Acta 92*, 421–432.

Boothe, R. L., & Folk, J. E. (1969) *J. Biol. Chem. 244*, 399–405.

Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331–4337.

Brockman, H. L., Jr. (1971) Ph.D. Thesis, Michigan State University, East Lansing, MI.

Brockman, H. L., & Wood, W. A. (1975) *J. Bacteriol. 124*, 1454–1461.

Casola, L., Brumby, P. E., & Massey, V. (1966) *J. Biol. Chem. 241*, 4977–4984.

Connellan, J. M., & Folk, J. E. (1969) *J. Biol. Chem. 244*, 3173–3181.

Cybulsky, D. L., Nagy, A., Kandel, S. I., Kandel, M., & Gornall, A. G. (1979) *J. Biol. Chem. 254*, 2032–2039.

Flashner, M., Hollenberg, P. F., & Coon, M. J. (1972) *J. Biol. Chem. 247*, 8114–8121.

Fonda, M. L. (1976) *J. Biol. Chem. 251*, 229–235.

Gale, G. R. (1961) *Arch. Biochem. Biophys. 94*, 236–240.

Glazer, A. N., & Smith, E. L. (1971) *Enzymes, 3rd Ed. 3*, 502–546.

Guggenheim, E. A. (1926) *Philos. Mag. 2*, 538–543.

Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, pp 94–109, Academic Press, New York.

Mulet, C., & Lederer, F. (1977) *Eur. J. Biochem. 73*, 443–447.

Okabe, K., Jacobs, H. K., & Kuby, S. A. (1970) *J. Biol. Chem. 245*, 6498–6510.

Olson, S. T. (1979) Ph.D. Dissertation, The University of Michigan, Ann Arbor, MI.

Olson, S. T., & Massey, V. (1979) *Biochemistry 18*, 4714–4724.

Olson, S. T., Massey, V., Ghisla, S., & Whitfield, C. D. (1979) *Biochemistry 18*, 4724–4732.

Riddles, P. W., Blakely, R. L., & Zerner, B. (1979) *Anal. Biochem. 94*, 75–81.

Spencer, R. L., & Wold, F. (1969) *Anal. Biochem. 32*, 185–190.

Thorpe, C., & Williams, C. H., Jr. (1975) *Biochemistry 14*, 2419–2424.

Yun, S., & Suelter, C. H. (1979) *J. Biol. Chem. 254*, 1811–1815.

Correlations between Tertiary Structure and Energetics of Coenzyme Binding in Pig Heart Muscle Lactate Dehydrogenase†

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ABSTRACT: Fluorescence, equilibrium dialysis, and microcalorimetric measurements have been performed on complex formation between pig heart muscle lactate dehydrogenase (EC 1.1.1.27) and a series of systematically modified nicotinamide adenine dinucleotide analogues to provide quantitative data for a discussion on energy–structure–function correlations. As a result of these studies, one can draw the conclusion that estimates of the relative stability of enzyme–ligand complexes on the mere basis of structural information on the macromolecule and its complexes with the ligand are likely to neglect contributions to the energy and entropy parameters, which stem from such processes as changes in solvation and conformation of both the free ligand and the macromolecule in the reaction. Since the reaction parameters reflect the differences between these states, information on hydrogen bonding and hydrophobic interaction schemes of the liganded and unliganded macromolecule alone is principally insufficient.

Detailed insight into the steric requirements of possible interactions between coenzyme and enzyme in the active center of dehydrogenases has been derived from comparative studies on various lactate dehydrogenase isozymes (Eventoff et al., 1977), on glyceraldehyde-3-phosphate dehydrogenases (Harris & Waters, 1976; Harris & Walker, 1977), and on alcohol dehydrogenase (Brändén, 1977; Brändén et al., 1975). Visualization of the tertiary structure does not, however, permit quantitative assessment of the energetic contributions of the various groups of the ligands involved in bond formation. Various approaches are possible in providing information on energetic details governing dehydrogenase–coenzyme complex formation. Two important approaches are (1) the utilization of a series of coenzyme fragments, the thermodynamic parameters of which are determined and compared to those of the natural coenzyme, and (2) the employment of the coenzyme itself which is modified chemically in a systematic fashion and analysis of the influence of the modifications on the overall energetic quantities. Results of studies on the energetics of binding of coenzyme fragments have been re-

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