ACTA CHEMICA SCANDINAVICA 10 (1956) 397—407

Essential Groups in DPN-linked Lactic Dehydrogenase

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Sulfhydryl groups in lactic dehydrogenase from ox heart (LDH) have been determined both amperometrically with mercuric chloride and spectrophotometrically with *p*-chloromercuribenzoate (PCMB). The enzyme appeared to contain 7—9 SH groups, but only 3 of these reacted with PCMB in the native enzyme. LDH was slowly inhibited by PCMB [4], but rapidly by mercury and silver ions; slowly by periodate, but rapidly by iodine, which oxidized SH groups. Incubation of LDH with acetic anhydride blocked amino groups, increased the reactivity of SH groups to PCMB and caused a strong inhibition. This inhibition was not reversed under conditions which hydrolyzed phenyl acetate and thiol acetate. Also a temporary inhibition by acetic anhydride was observed, which existed while acetic anhydride was present in the solution.

The great majority of DPN-linked ** enzymes contain essential sulfhydryl groups which are susceptible to most SH reagents [1]. In contrast, DPN-linked lactic dehydrogenase from ox heart (LDH) is not readily inhibited by a number of SH reagents [2,3], and was for that reason believed not to require SH groups for activity. Recently, however, *p*-chloromercuribenzoate (PCMB) has been found to react slowly with the enzyme, thus causing inhibition [4]. In this work the reactivity and essentiality of SH groups in LDH have been further investigated.

Many enzymes are known to depend on SH groups for their activity. Less is known about other kinds of functional groups. Ideal reagents are lacking in many cases. Further work may reveal other groups as frequently involved in the regulation of enzyme activity as are SH groups. In this study, the essentiality of amino groups in LDH has been investigated. As carriers of positive charge, these groups could be expected to play a role in the electrostatic interaction of enzyme and coenzyme or substrate. Extensive studies have revealed

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** The following abbreviations are used: DPN: diphosphopyridine nucleotide, DPNH: reduced diphosphopyridine nucleotide, LDH: DPN-linked lactic dehydrogenase from ox heart, PCMB: *p*-chloromercuribenzoate and SH: sulfhydryl (thiol) group.

this kind of interaction between flavin mononucleotide and the apoprotein of the old yellow enzyme [5].

Also included in this work is the detection of what appears to be a very labile acetyl compound of LDH.

A short account on the work with SH groups in LDH has already been published [6].

MATERIALS AND METHODS

Crystalline LDH, containing fractions A and C [3,4] was prepared according to Straub [7]. The enzyme concentration was determined from optical density measurements at 280 mμ. In the calculation, a molar extinction coefficient of 20.1×10^4 and a molecular weight of 135 000 were used [3,4].

Crystalline liver alcohol dehydrogenase was a gift from Dr. R. Bonnichsen.

DPN, 90 % pure, was procured from Sigma Chemical Company, St. Louis, Mo.

DPNH was prepared by enzymatic reduction of DPN [8].

Sodiumpyruvate was made from commercial pyruvic acid [9].

DL-*Lactic acid* was Baker's analyzed reagent.

Oxidoreduction of DPN(H) was measured with a fluorimeter of the type described elsewhere [10, 11].

Iodination of the enzyme was carried out at 0° C. The iodinating agent was 0.1 M iodine dissolved in 96 % alcohol.

Acetylation of the enzyme and of cysteine and tyrosine was accomplished by the addition of 1 μl quantities of acetic anhydride to approximately 1 ml of the protein (amino acid) solution buffered with 2.5 M sodium acetate and kept at 0° C [12]. To the control the same amount of acetic anhydride was added to the acetate solution half an hour before the enzyme (amino acid) was added.

Thiol groups were determined both spectrophotometrically with PCMB according to Boyer [13] and amperometrically according to Kolthoff *et al.*[14] In the spectrophotometric determinations the change in optical density at 250 mμ was recorded and a molecular extinction coefficient of 7.6×10^3 was used to calculate the extent of the reaction. Spectrophotometric determinations were carried out both with the native and denatured enzyme. Denaturation was accomplished by incubation of the enzyme in 10 % lauryl sulfate. Foaming prevented amperometric titration of a protein denatured with lauryl sulfate. The amperometric titration had to be carried out within 10 minutes, otherwise the potentials drifted and measurements were impossible. Cysteine treated with acetic anhydride gave no reaction with PCMB or with mercury ions in the amperometric titration. This shows that the thiol groups were blocked and that the acetyl-thiol linkage was stable under the conditions of analysis. Cysteine treated with acetic anhydride will be designated S-acetyl cysteine. Undoubtedly, the amino group was also acetylated in this compound.

Tyrosine was estimated by the Millon-Lugg procedure [15], and the diiodotyrosine formed was calculated from the difference in tyrosine content determined by this procedure before and after iodination [12].

Amino groups were determined by Van Slyke amino nitrogen analysis [16], which was carried out on dialyzed samples containing approximately 15 mg of protein. A 15 minute reaction time was found sufficient to give maximum values.

Phenol groups were estimated by means of Folin's phenol reagent [17], the protein first being denatured with lauryl sulfate [18]. When tyrosine had been treated with acetic anhydride the color reaction was negative, indicating that phenolic hydroxyl was acetylated and that the linkage was stable under the conditions of analysis. Tyrosine treated with acetic anhydride is designated O-acetyl tyrosine. This compound was undoubtedly also acetylated in the amino group.

Hydrolysis of phenol acetate and thiol acetate can be accomplished by incubation at pH 11 for about 5 minutes [12]. The acetyl-amino linkage is stable under these conditions. Therefore, if no reactivation of an acetylated enzyme takes

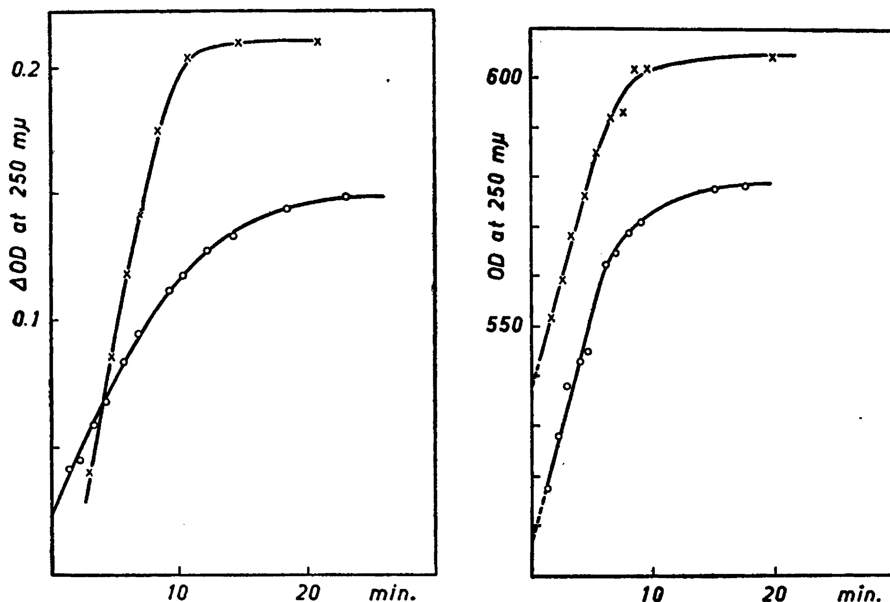

Fig. 1. Reaction of LDH with p-chloromercuribenzoate, before and after treatment of the enzyme with acetic anhydride.

Test conditions: To 2.6 ml 0.2 M phosphate buffer were added 94 mμmoles of PCMB and 14.5 mμmoles of LDH. Final volume 3.0 ml. For unmodified enzyme Δ OD = 0.108

Therefore $\frac{0.108 \times 3 \times 10^{6}}{7.6 \times 10^{3} \times 14.5} = 2.9$ SH groups per mole enzyme.

LDH was acetylated as follows: To 20.1 mumoles LDH in 0.4 ml 2.5 M sodium acetate pH 7 were added 5 × 1 μl of acetic anhydride at 0° C with one minute's intervals. 290 μl of the solution was allowed to react with PCMB. The enzyme activity decreased 45 % by the acetylation. × × × acetylated LDH O O O LDH, unmodified.

In order to measure total thiol groups, the enzyme dissolved in a few μl solution was allowed to react with 100 μl of 10 % lauryl sulfate before the addition of buffer and PCMB. The denatured enzyme reacted instantaneously with PCMB, as also did cysteine.

Fig. 2. Reaction of liver alcohol dehydrogenase (ADH) with p-chloromercuribenzoate before and after treatment with acetic anhydride.

Test conditions: To 2.66 ml 0.2 M phosphate buffer were added 159 mμmoles PCMB and 8.7 mμmoles ADH. Final pH 6.3. Total volume 3.0 ml Δ OD = 0.070. Therefore

$\frac{0.07 \times 3 \times 10^{6}}{7.6 \times 10^{3} \times 8.7} = 3.1$ SH groups per mole ADH.

ADH was acetylated as follows: To 32.6 mμmoles enzyme in 0.4 ml of 2.5 M sodium acetate pH 7 was added 5 × 1 μl acetic anhydride at 0° C with one minute intervals. 106 μl of the solution was allowed to react with PCMB. The enzyme activity decreased 62 % by the acetylation. None of the activity was recovered by incubation in 1 M glycine pH 9.5. × × × acetylated ADH O O O ADH, unmodified

place with incubation at pH 11, essential phenol or thiol groups have probably not been acetylated [19]. However, incubation at pH 11 may cause denaturation of some proteins. We have found that high alkalinity can be avoided by the use of high concentrations of glycine. Thus, O-acetyl tyrosine and S-acetyl cysteine

were found to be hydrolyzed within a few minutes by incubation in 1 M glycine at pH 9.5 but not affected by 0.1 M glycine at pH 9.5. The evidence was that Folin's phenol color (for tyrosine) and the reaction with PCMB (for cysteine) reappeared. LDH, liver alcohol dehydrogenase and the apoenzyme of the old yellow enzyme were perfectly stable in 1 M glycine pH 9.5. The acetyl-amino linkage in acetylated LDH was not hydrolyzed under these conditions, as there was no increase in Van Slyke amino group analysis.

RESULTS

Neilands [3] observed that 10^{-3} M PCMB had no instantaneous effect on LDH activity. However, incubation with the reagent caused inhibition, which was reversed by cysteine [4]. These observations have been confirmed in this study. The reaction between PCMB and LDH, reflecting the inhibition of the enzyme, is shown in Fig. 1. Under the conditions used, 2.8 SH groups reacted with PCMB, whereas Neilands [4] recorded 2 SH groups combining. Also included in Fig. 1 is the more extensive and more rapid reaction between PCMB and an acetylated form of the enzyme. Evidence for specific acetylation of amino groups will be presented later. In contrast to LDH, an acetylated form of liver alcohol dehydrogenase and the unmodified enzyme reacted similarly

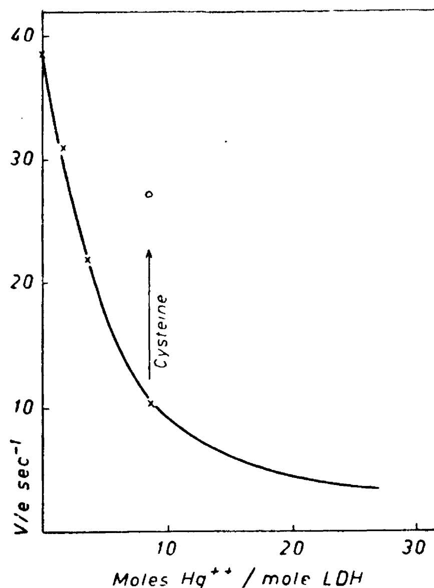

Fig. 3. Inactivation of LDH by $HgCl_2$.

10^{-2} or 10^{-3} M $HgCl_2$ was added to 1.14 mμmoles LDH in 25 μl of 0.02 M phosphate pH 7.2 and the mixture was allowed to react for 2 minutes at 0° C. The solution was then diluted to 500 μl with water and 5 μl were used for testing of activity in 3.0 ml of 0.1 M glycine pH 9.0. Conc. DL-lactate: 8.3 μM. Conc. DPN: 420 μM. To one sample (see figure) 10 μl of 0.5 M neutralized cysteine were added after dilution to 500 μl. The inhibition did not increase with a longer incubation time than 2 minutes.

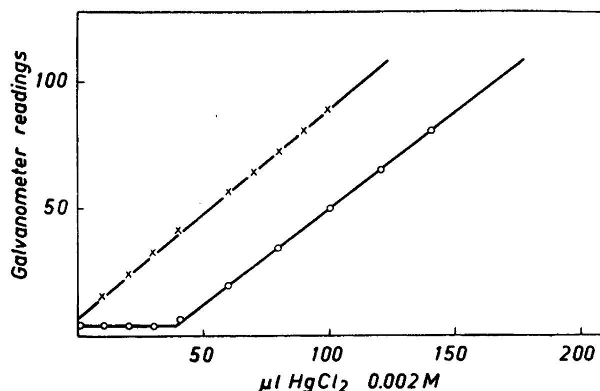

Fig. 4. Amperometric titration of LDH with $HgCl_2$.

The buffer used was 0.01 M in NaH_2PO_4, 0.08 M in Na_2HPO_4 and 0.5 N in KCl [14]. The titration was carried out within 10 min. 11 mμmoles LDH were found to bind 39 μl of 0.002 M $HgCl_2$. That is, 7.1 SH groups per mole LDH reacted with mercuric chloride. × × × blank O O O 11 mμmoles LDH.

with PCMB. This is shown in Fig. 2. For LDH denatured with lauryl sulfate, the reaction with PCMB was too rapid to be followed in a Beckman spectrophotometer; a total of 9.3 SH groups appeared to react.

In contrast to the slow inhibition of LDH by PCMB, mercuric and silver salts have been found to cause a very rapid inactivation of the enzyme. Thus, when 0.018 mμmole of LDH was added to 3.0 ml glycine buffer at pH 9.0, which was 8.3 mM in DL-lactate, 300 μM in DPN and 0.33 μM in Ag_2SO_4, no reduction of DPN was observed. The enzyme must have been inactivated within a few seconds. Inactivation of LDH as a function of added mercuric chloride is illustrated in Fig. 3. The incubation time was 2 minutes. Under the conditions used, 10 moles of mercury per mole of enzyme caused 75 % inhibition. A similar result was observed with Ag_2SO_4. Only 95 % inhibition was obtained with heavy metal ions some five-fold in excess of the total number of thiol groups. Cysteine counteracted the inhibition by $HgCl_2$ and Ag_2SO_4 to varying degrees, apparently depending upon the conditions of the incubation. In one case, a sample which had been completely inhibited by Ag_2SO_4 was reactivated completely. Fig. 4 shows the results of an amperometric titration [14] of LDH with $HgCl_2$ whereby 7 thiol groups were titrated. This is 2 SH groups less than obtained with the spectrophotometric titration with PCMB.

Incubation with iodine resulted in a rapid decrease of the LDH activity. The inhibition illustrated in Fig. 5 was registered within a few minutes after the addition of the reagent, which rapidly lost its color. The following observations suggest strongly that the inhibition was due to oxidation of essential SH groups, mainly to the disulfide stage, and not to substitution of tyrosine [12]: 1) No diiodotyrosine was formed, even in the most iodinated sample (See Fig. 5). 2) The reaction of LDH with PCMB disappeared with iodination of the protein. 3) The amount of iodine necessary for complete inactivation corres-

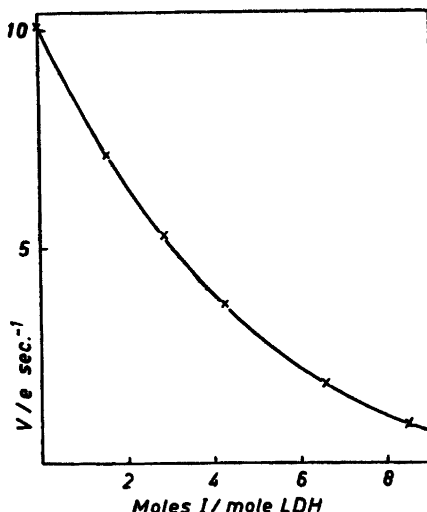

Fig. 5. Inactivation of LDH with iodine.

To 28.2 mμmoles LDH in 0.7 ml of 0.1 M phosphate pH 7.3, 0° C, were added 1 μl quantities of 0.1 M I in alcohol with 5 minute's intervals. To the controls corresponding amounts of alcohol were added. 2 μl of the enzyme solution were used for testing in 3.0 ml of 0.1 M glycine buffer pH 9.0 at 23° C. Conc. DL-lactate: 2.6 mM and conc. DPN 108 *μM.*

25.8 moles of tyrosine per mole LDH were found with the Millon-Lugg analysis [15] in the most iodinated sample as well as in the control. However, when LDH was iodinated in 0.1 M glycine pH 9.2, 13 % of the iodine was used for substitution of tyrosine to form diiodotyrosine in the most iodinated sample.

ponded roughly to the total number of thiol groups (Fig. 5). 4) Cysteine reactivated the iodinated enzyme. This has also been observed by Neilands [4].

In contrast to the rapid inactivation with iodine, the strong oxidizing reagent, periodate, affected the enzyme only slowly, thus showing that the essential SH groups were unreactive to this reagent. When 5.9 mμmoles LDH were incubated at pH 5.5 with 50 mμmoles periodate in 110 μl 0.6 M acetate, only 25 % of the activity was lost after 1.5 hours incubation. The inactivation took place continuously. Liver alcohol dehydrogenase was inactivated within a few minutes under the same conditions.

The observations reported in the literature [2,3] that LDH is not inhibited by O-iodosobenzoate or iodoacetate have been confirmed.

Acetylation of LDH with acetic anhydride inactivated LDH strongly. Table 1 shows the activity of acetylated LDH as a function of free amino groups. Acetic anhydride has been claimed to be specific for amino groups in all proteins tested [12]. However, the reagent does not necessarily react in the same manner with all proteins. The blocking of SH and phenolic hydroxyl groups appeared as a possibility because ketene, which is a stronger acetylating reagent than is acetic anhydride [12], has been found to acetylate these groups in some proteins. Moreover, acetic anhydride forms O-acetyl tyrosine and S-

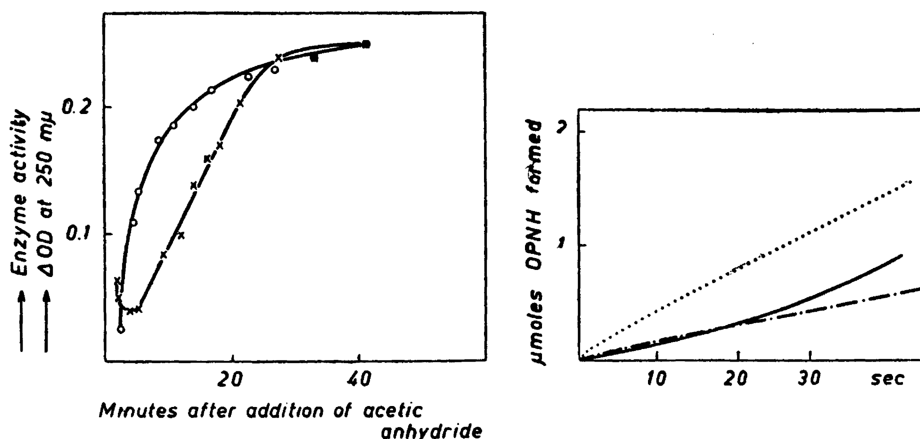

Fig. 6. Reactivation of acetylated LDH and change of optical density of the solution.

a. To 11.1 mμmoles LDH in 0.19 ml buffer (2.5 M in acetate, 0.1 M in Na_2HPO_4) were added 5 × 1 μl acetic anhydride with some seconds intervals. Temp. 0° C. Immediately after the last addition of acetic anhydride, 3.11 ml phosphate buffer (μ = 0.1, pH 7.13) were mixed with the enzyme solution. Final pH: 6.45, temp. 23° C. The change of optical density of this solution at 250 mμ was followed in a Beckman spectrophotometer. Activity measurements of aliquots of the same solution were carried out simultaneously in 0.1 glycine pH 9.75. Conc. DPN: 400 μM and conc. DL-lactate 1.77 mM.

O O O Change in OD_{250} mμ.

× × × Enzyme activity.

b The same experiment as *a*, but now illustrating the activity increase during lactate oxidation, instead of initial velocities used in *a*. ———— 2.5 min. —·—·— 8.5 min. ·········· 41 min. after the last addition of acetic anhydride.

acetyl cysteine. Now, if essential SH or phenolic hydroxyl groups in LDH were acetylated, incubation in 1 M glycine at pH 9.5 should reactivate the enzyme to the same degree as these groups were involved (see Methods). But no reactivation of acetylated LDH took place provided the samples were tested at least half an hour after the addition of acetic anhydride *. Furthermore, the Folin's phenol color was not significantly decreased in the acetylated sample, and amperometric titration gave the same result before and after acetylation, around 7 SH groups being titrated in both cases. Therefore, neither phenyl acetate nor thiol acetate appeared to be formed with acetylation of LDH, thus indicating that acetic anhydride was also specific for amino groups in LDH.

Preliminary experiments with formaldehyde and phenylisocyante showed that LDH is sensitive to these amino group reagents. Thus, incubation of a 0.8 % solution of LDH with 0.25 M formaldehyde at pH 7.4 (0° C) decreased the activity by 38 % in one minute and 47 % in 10 minutes. Incubation of 65 μl 0.8 % LDH with 1 μl phenylisocyanate at pH 7.4 (0° C) decreased the activity by 89 % in 3 minutes.

As mentioned above, no reactivation of acetylated LDH took place by incubation with 1 M glycine pH 9.5 provided the samples were tested at least

* For further elaboration on this point, see below.

half an hour after the addition of acetic anhydride. If, however, the activity was tested immediately after the addition of acetic anhydride, reactivation took place within one minute in 1 M glycine at pH 9.5, and within one hour if the enzyme remained in the acetate solution. The same level of enzymatic activity was reached in both cases, some inhibition remaining due to acetylation of amino groups. Fig. 6a shows the reactivation of the enzyme in 2.5 M sodium acetate pH 6. Under these conditions, O-acetyl tyrosine and S-acetyl cysteine were stable. The initial decrease in activity shown in Fig. 6a is probably due to continued acetylation when relatively large amounts of acetic anhydride were still present in the solution. Fig. 6b illustrates reactivation of the same acetylated enzyme during oxidation of DL-lactate in 0.1 M glycine at pH 9.7. A similar reactivation of acetylated LDH was observed during reduction of pyruvate. In an attempt to discover spectral changes of the protein as the activity was restored, we observed that acetic anhydride itself caused a strong spectral absorption in the 250 mμ region, which disappeared upon hydrolysis. In Fig. 6a is included the spectral change that occurred simultaneously with the reactivation. A similar spectral change took place with acetic anhydride in the absence of protein. From the figure it is apparent that reactivation occurs until all acetic anhydride has disappeared from the solution, thus suggesting that this compound prevents the hydrolysis of a labile acetyl compound of LDH.* Also liver alcohol dehydrogenase ** and the apoenzyme of the old yellow enzyme [20] have been incubated with acetic anhydride. Although both proteins were strongly affected by the reagent, apparently by specific acetylation of amino groups, neither of them behaved like LDH with respect to temporary inhibition of acetic anhydride. The activity of liver alcohol dehydrogenase as well as the recombination rate of the apoprotein and FMN were not increased when acetic anhydride disappeared from the solution.

DISCUSSION

The requirements of an ideal inhibitor are that it should react rapidly and specifically with a protein group, and that it should be possible to regenerate the enzyme after treatment with the inhibitor [19]. Silver and mercury ions appear to be ideal inhibitors for SH groups in LDH. PCMB, on the other hand, reacts too slowly, and some of the effects of this reagent may be due to structural alteration. Therefore, the observation that only half or less of the SH groups reacted with PCMB to cause almost complete inactivation [4] does not necessarily imply that the essential SH groups are more reactive to PCMB than are the other SH groups. In the case of mercury and iodine inactivation, the data presented in Figs. 3 and 5 indicate the essential groups to have the same reactivity as the other SH groups toward these reagents.

* Experiments with crystalline LDH from sceletal muscle (C. F. Boehringer & Soehne, Mannheim) and with 80 % pure rat liver LDH (a gift from Dr. Carl S. Vestling, Un. of Illinois) have shown that these enzymes behave in the same manner as ox heart LDH regarding temporary inhibition by acetic anhydride.

** No reactivation of acetylated liver alcohol dehydrogenase took place in 1 M glycine at pH 9.5. See Fig. 2.

Table 1. The decrease of LDH activity with acetylation of amino groups.

pH	Number of free amino groups			
	97 *	76	67	58
	E/V sec	% of the activity of unmodified		
7.1	8.6	57	38	30
9.0	19.7	61	31	29
9.5	25.7	66	36	36

To 95 mμmoles LDH in 0.8 ml of 2.5 M acetate pH 7 were added 1, 3 × 1 or 4 × 1 μl of acetic anhydride at 0° C. The activity was tested one hour after the addition of acetic anhydride, in phosphate buffer (μ = 0.1) pH 7.15, in 0.1 M glycine pH 9.0 and in 0.1 M glycine pH 9.5. Conc. DL-lactate: 1.7 mM. Conc. DPN: 415 μM.

The findings that 10 moles of mercury or silver ions per mole of enzyme caused only 75 % inhibition, whereas the number of thiol groups appear to be 7—9, could be the result of high dilution of the enzyme in the activity measurements. Thus, in the titration experiments with mercury the enzyme concentration was about 10^{-6} M, whereas the final dilution in the activity measurements was approximately 10^{-9} M. If a rapid equilibrium were established between SH and the mercury ions, the above discrepancy could perhaps be accounted for in terms of dissociation of the mercaptide. The difference between 7 SH groups per mole enzyme titrated with mercuric chloride and 9.3 SH groups determined spectrophotometrically with PCMB remains unexplained.

The rapid reaction of silver and mercury ions and of iodine with SH groups in LDH, the slow reaction of PCMB and periodate, and the inertness to such SH reagents as O-iodosobenzoate, iodoacetate and N-ethylmaleimide may reflect different permeabilities of molecules of different sizes to the masked SH groups.

The fact that specific acetylation of amino groups inactivated LDH suggest that amino groups are essential for activity. This conclusion is supported by the observation that LDH is inactivated by formaldehyde and phenylisocyanate under conditions where these reagents are fairly specific for protein amino groups. If it is assumed that the extent of inhibition represents the fraction of essential groups which have been acetylated, then the essential amino groups are more reactive to acetic anhydride than are the other amino groups [20]. Table 1 shows that the activity decreases more rapidly than would have been the case if the blocking took place at random. However, the finding that PCMB reacted faster with acetylated enzyme than with the control shows that acetylation beside blocking amino groups also may alter the protein structure so as to increase the reactivity of thiol groups. Unfortunately, the reversibility of this alteration could not be determined. The

* Unmodified.

acetyl-amino linkage can hardly be hydrolyzed without denaturation of the enzyme. The alteration is not likely to be surface denaturation in view of the stability of other proteins to acetic anhydride [12], including liver alcohol dehydrogenase (see Fig. 2).

The activity measurements of acetylated LDH at different hydrogen ion concentrations recorded in Table 1 indicate that the decrease in activity is not due to a general discharge of the protein molecule. At pH 9.5 the number of positive charges of the enzyme should be much less than at pH 7.1, but the relative decrease in activity was roughly the same whether the activity was determined at pH 9.5, 9.0 or 7.1.

The observation that acetic anhydride effected a temporary inactivation of LDH may be explained if it is assumed that essential groups gave readily hydrolyzable acetates. The fact that the activity was regained much faster in 1 M glycine at pH 9.5 than in neutral acetate solution supports this conclusion. N-acetylimidazole has been shown to be a very labile compound [21], and it appears possible that an essential imidazol group in LDH has been acetylated. However, there are other possible explanations. Although S-acetyl cysteine and O-acetyl tyrosine are more stable than the acetyl compound of LDH, these groups may nevertheless be involved. Thus, the sluggishly reactive SH groups could form an acetyl compound more labile than S-acetyl cysteine. Unfortunately, because of the lability of the acetyl LDH compound, measurements for the blocking of these groups were impossible.

The suggestion has been made that thiol groups are involved in the binding of coenzyme or substrate in some DPN-linked enzymes [1]. In view of the low reactivity of SH groups in LDH to PCMB, periodate, iodosobenzoate, iodoacetate and N-ethylmaleimide these groups may not be sufficiently reactive to bind such relatively large molecules as lactate, pyruvate, DPN or DPNH. However, participation of amino groups in the binding could facilitate binding to the SH group(s), in the same way as the reactivity of SH groups increases when amino groups are acetylated. Such an interaction would require a compulsory sequence of reactions between protein, coenzyme and substrate.

Thanks are due to professor Hugo Theorell for his interest in this work, to Dr. Ryo S. Sato for a sample of LDH which started out this work, and to Miss Ann Louise Swanson for valuable technical assistance. A personal grant from *A/S Freias Chocolade Fabriks arbeidsfond for ernæringsforsking* is gratefully acknowledged.

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Received November 18, 1955.