SELECTIVE CARBOXYMETHYLATION OF FUNCTIONAL SULFHYDRYL GROUPS AT THE ACTIVE CENTER OF HORSE LIVER ALCOHOL DEHYDROGENASE *

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Crystalline horse liver alcohol dehydrogenase (LADH) has variously been reported to contain 20-28 sulfhydryl groups, which are thought to be essential for the maintenance of protein structure (Witter, 1960; Li et al., 1962). Analytical evidence for their postulated participation in the enzymatic process has been lacking thus far.

We have found that iodoacetate inactivates the enzyme through preferential alkylation of approximately 2 of the -SH groups of the apoenzyme without gross alteration of protein structure. DPN(H) protects the enzyme almost completely against the chemical and enzymatic changes induced by iodoacetate.

Experimental

Horse liver alcohol dehydrogenase (C.F. Boehringer and Soehne, Mannheim, West Germany) in 0.1 M phosphate, pH 7.5 was incubated

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with 600 fold molar excess of cold and C^{14}-labelled iodoacetate at 21° for 2 to 2 1/2 hours. Enzymatic activity was assayed as described previously (Ulmer and Vallee, 1961). The reaction was terminated by passage of the solution over Sephadex G-25 to separate excess iodoacetate from the enzyme. When iodoacetate-1-C^{14} incorporation and amino acid composition were to be measured, the protein fraction was further dialyzed against water at pH 7.5, 10° for 18 hours. The -SH titer of the enzyme was measured by titration with PCMB (Boyer, 1954). Measurement of hydrogen ions liberated during carboxymethylation was performed in 0.05 M NaCl under nitrogen in a pH-stat regulated at pH 7.5 (Benesch and Benesch, 1962). Incorporation of iodoacetate-1-C^{14} into the enzyme was determined by standard procedures of liquid scintillation counting. Sedimentation was performed in a model E Spinco Ultracentrifuge. Optical rotatory dispersion was measured as described previously (Li et al., 1962).

Results

Exposure of the LADH·DPNH complex to 600 fold molar excess of iodoacetate at pH 7.5, 21° does not impair enzymatic activity significantly (Figure 1). Over a period of 2 1/2 hours, about 0.3 protons are liberated and 0.8 moles of C^{14}-carboxymethyl groups are incorporated per mole of protein. Simultaneously, less than one mole of S-carboxymethylcysteine is formed [1] and the sulfhydryl titer of the enzyme complex decreases from 23.1 to 22.5 moles -SH per mole of protein (Table I, Column 1).

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1 We are greatly indebted to Dr. Christian B. Anfinsen for these analyses.

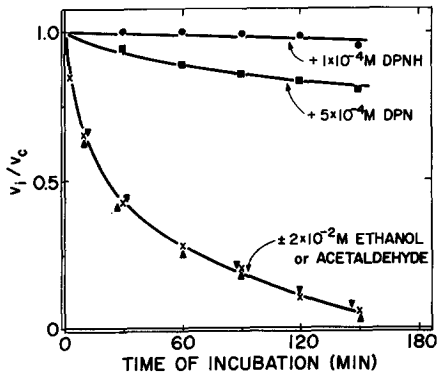

Figure 1. Effect of coenzymes and substrates upon the time-dependent inhibition of LADH by iodoacetate. Partial specific activity, V_i/V_c, is plotted against time of incubation. 1×10^{-5} M LADH in 0.1 M phosphate, pH 7.5, 21° is incubated with 6×10^{-3} M iodoacetate in the presence of 1×10^{-4} M DPNH (●), 5×10^{-4} M DPN (■), 2×10^{-2} M ethanol (▼), 2×10^{-2} M acetaldehyde (▲), and in their absence (X). Aliquots are diluted, and enzymatic activity (DPN→ DPNH) is assayed at the indicated times.

Table I. Reaction of Iodoacetate with LADH·DPNH and LADH

(LADH, 6×10^{-5} M; DPNH, 1.5×10^{-3} M; Iodoacetate, 3.6×10^{-2} M; pH 7.5, 21°)

	LADH·DPNH	LADH	DIFFERENCE
Enzymatic activity (% of control)	95	0	95
Protons released per mole protein	0.2 0.4	2.1 2.6	1.9 2.2
C^{14}-carboxymethyl incorporated per mole protein	0.8	3.3	2.5
-SH groups per mole protein*	22.4 22.6	20.2 20.8	2.2 1.8

* The -SH titer of the native enzyme employed was 23.1 moles -SH per mole of protein

In contrast, reaction of iodoacetate with the apoenzyme under identical conditions progressively and irreversibly inactivates the enzyme at a first order rate until less than 5% of the original activity remains after 2 1/2 hours (Table I, Column 2; Figure 1). The difference between the values in columns 1 and 2 of table I demonstrates that during the course of the inhibition, 2 additional protons are liberated and 2.5 C^{14}-carboxymethyl groups are incorporated per mole of protein. As a result, 2 moles of -SH groups per mole of protein are blocked as discerned by titration with PCMB (Table I, Column 3). Preliminary amino acid analysis[1] after acid hydrolysis of the carboxymethylated enzyme, Cm-LADH, demonstrates a difference of about 2 moles of S-carboxymethyl-cysteine.

DPN, which has a weaker affinity for LADH than does DPNH, (Vallee et al., 1959) also protects the apoenzyme against inhibition by iodoacetate, while ethanol and acetaldehyde do not (Figure 1).

In spite of the profound effects on activity, upon carboxymethylation there is virtually no change in specific rotation, optical rotatory dispersion and the sedimentation coefficient, parameters commonly employed to detect alterations of protein structure and conformation (Table II).

The interaction of DPNH with the inactive, carboxymethylated enzyme was studied spectrally and by Rotatory Dispersion Titration (Theorell and Bonnichsen, 1951; Li et al., 1962). Cm-LADH still binds 2 moles of DPNH, albeit less firmly.

Table II. Physical Properties of Native and Carboxymethyl-LADH (Cm-LADH)

	$-[\alpha]^{10}_{420}$ (degrees)	λc (mμ)	$s_{20,b}$ (S)
native LADH	51	243	4.4*
Cm-LADH	49	244	4.5+

$[\alpha]$, λc and $s_{20,b}$ were measured as indicated under methods

* 7.4 mg/ml protein in 0.1 M PO_4, pH 7.5, and 1% KCl
\+ 5.1 mg/ml protein in 0.1 M PO_4, pH 7.5, and 1% KCl

The dissociation constant of the Cm-LADH·DPNH complex is 3×10^{-5} M.

Discussion

Previous studies designed to elucidate the role of sulfhydryl groups in the mechanism of action of LADH have shown that chemical modification of the enzyme with a variety of "-SH reagents" may result both in loss of enzymatic activity and in alterations of protein structure (Witter, 1960; Li et al., 1962; Yonetani and Theorell, 1962). Attempts to differentiate groups essential for activity from others required for the maintenance of protein structure have met with limited success.

Under the conditions here employed, iodoacetate preferentially carboxymethylates a limited number of cysteine residues leading to complete inactivation of the enzyme but without measurable changes in its protein structure. DPNH prevents both these enzymatic changes and the modification of approximately 2

-SH groups. Hence, the loss of enzymatic function can be attributed to their carboxymethylation.

Alkylation of other -SH groups, apparently non-essential for activity, may also occur during the reaction, although their rate of reaction with iodoacetate is much slower and varies as a function both of experimental conditions and of different enzyme preparations (Table I, Column 1). Subtraction of the number of groups modified non-specifically from the total number of the apoenzyme which are alkylated yields 1.8 to 2.5 -SH groups per mole of protein essential for activity, an average of one -SH group per active, enzymatic site. Efforts to further reduce the reaction of non-essential -SH groups and to isolate peptides containing the reactive cysteine residues are under way.

The two iodoacetate reactive -SH groups may participate in the catalytic process, DPN(H) binding or both. Studies in progress are designed to provide a solution to this problem.

REFERENCES

Benesch, R. and R.E. Benesch in Methods of Biochemical Analysis Vol. 10, D. Glick, Ed. Interscience Publishers, 1962, p.43
Boyer, P.D., J. Am. Chem. Soc. 73, 4331 (1954)
Li, T.K., D.D. Ulmer,and B.L. Vallee, Biochemistry 1, 114 (1962)
Theorell, H. and R. Bonnichsen, Acta Chem. Scand. 5, 1105 (1951)
Ulmer, D.D. and B.L. Vallee, J. Biol. Chem. 236, 730 (1961)
Vallee, B.L., R.J.P. Williams, and F.L. Hoch, J. Biol. Chem. 234, 2621 (1959)
Witter, A., Acta Chem. Scand. 14, 1717 (1960)
Yonetani, T. and H. Theorell, Arch. Biochem. & Biophys. 99, 433 (1962)