STRUCTURE-FUNCTION STUDIES ON GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. III. DEPENDENCY OF PROTEOLYSIS ON NAD^+ CONCENTRATION*

Allan Fenselau
Department of Physiological Chemistry, The Johns Hopkins University
School of Medicine, Baltimore, Maryland 21205

Received June 22, 1970

Summary: Mild treatment of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with chymotrypsin, trypsin, subtilisin, or Pronase results in a substantial loss in enzymatic activity of the apoenzyme and not the holoenzyme. The products from proteolytic digestion differ in chemical and physical properties, suggesting that peptide bond cleavages have been introduced in quite different regions of the protein. Particularly in the cases of chymotrypsin and trypsin binding of NAD^+ drastically reduces the susceptibility of all these bonds to hydrolysis. The degree of protection from inactivation is greatest in the case of all proteolyses when less than two coenzyme molecules are bound to a molecule of the tetrameric enzyme. These results are considered in terms of understanding the mechanism for the homotropic effects observed in coenzyme binding.

Controlled digestion with chymotrypsin of apoglyceraldehyde-3-phosphate dehydrogenase[1] from rabbit muscle leads to a loss in the physiological enzymatic activity, the oxidative phosphorylation of glyceraldehyde-3-phosphate; however, the holoenzyme under the same conditions shows no such effect (1,2). By analogy to the studies of McClintock and Markus (3) on aspartate transcarbamylase these observations suggest that information on changes in enzyme quaternary structure with ligand binding might be afforded by these proteolytic methods. The objectives of this investigation were to examine the generality of this effect of coenzyme binding on GAPDH digestibility using other proteases and then to determine the dependency of this effect on NAD^+ concentration. The latter results would consequently apply toward elucidating a mechanism for the homotropic

*This investigation was supported by a National Science Foundation grant (GB 7326) and an institutional research grant from the National Institutes of Health.

[1] Abbreviations used: G-3-P, D-glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase: G-3-P; NAD^+-oxidoreductase (phosphorylating) EC 1.2.1.12.

effect observed in coenzyme binding (4,5).

Materials and Methods - Crystalline rabbit muscle GAPDH was obtained from Sigma Chemical Corp. as were Pronase (the fungal protease from S. griseus), subtilisin (the bacterial protease from B. subtilis), NAD^+, and G-3-P. α-Chymotrypsin (analytical grade) and trypsin (1x crystallized) were purchased from Boehringer-Mannheim Corp. and Mann Research Lab., Inc., respectively. All other chemicals are reagent grade. Procedures for assaying enzymatic activity (6) and for determining the extent of incorporation of ^{14}C-carboxymethyl groups (7) have been described elsewhere. Sodium dodecyl sulfate (SDS) disc gel electrophoresis was carried out according to the method of Weber and Osborn (8). Apo-GAPDH was prepared by batchwise charcoal treatment ($A_{280}:A_{260} > 1.85$). The extinction coefficients of Fox and Dandliker (9) were used for determining protein concentration (mol. wt. for apoenzyme, 144,000).

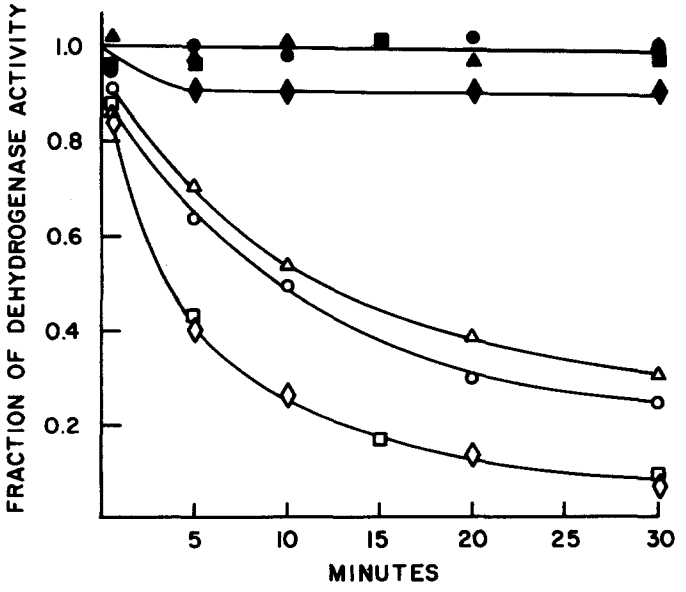

Figure 1. Change in Rabbit Muscle GAPDH Enzymatic Activity during Limited Proteolysis in the Presence (closed symbols) and Absence (open symbols) of NAD^+. The proteases employed were chymotrypsin (○, 0.025%), subtilisin (△, 0.025%), trypsin (□, 0.025%), and Pronase (◇, 0.025%). In all cases apo-GAPDH (91 μM) was incubated with the proteolytic enzyme at 25°C in a Tris-HCl solution (0.05 M, pH 8.0 with 1 mM EDTA and 0.20 M $(NH_4)_2SO_4$). The concentration of NAD^+ (when present) was 2.0 mM.

Results - In Fig. 1 is shown the effect of various proteases on enzymatic activity when apo-GAPDH is digested at 25°C in the absence and presence of NAD^+. In all cases the enzyme is considerably more susceptible to inactivation when coenzyme is absent. When conditions with chymotrypsin as reported previously (1,2) are employed, the same leveling out in activity at <30% is observed. Both trypsin and the non-specific Pronase (10) rapidly reduce activity to approximately 5% of the original value. NAD^+ had no

TABLE I

Incorporation of ^{14}C-Carboxymethyl Groups into Protease-modified GAPDH

To a solution of apo-GAPDH (87 μM in 0.05 M Tris-HCl at pH 8.0 containing 1 mM EDTA and 0.20 M $(NH_4)_2SO_4$) either in the absence or presence of NAD^+ (initial conc., 3.8 mM) was added the protease (25 μg). After 30 min. at 25°C followed by a dehydrogenase assay the enzyme solution was diluted with Tris-HCl buffer (pH 6.0) containing ^{14}C-iodoacetate (^{14}C-IAA; sp.act. 9.0 x 10^5 dpm μmole^{-1}) (with or without NAD^+) such that the final concentrations were : GAPDH, 49 μM; NAD^+, 2.2 mM; ^{14}C-IAA, 0.40 mM; and protease, (15 μg) (final pH 7.1). Fifty microliters of this solution were plated at 10 min. on a glass fiber disc and processed for scintillation counting (7).

Proteolytic Incubation with	Number[a] of ^{14}C-Carboxymethyl Groups Incorporated after 10 min.	
	APO[b]	HOLO[b]
Control (no protease)	1.4 (100)[c]	3.6 (100)
Chymotrypsin	2.7 (25)	3.3 (98)
Trypsin	1.8 (10)	3.2 (98)
Pronase	1.8 (15)	3.0 (94)
Subtilisin	1.5 (34)	3.0 (99)

a) Determined on the basis of an assumed mol.wt. of 144,000 for both modified and unmodified GAPDH.

b) Apo and Holo refer to the state of GAPDH during proteolysis; during treatment with ^{14}C-IAA the NAD^+ concentration was 2.2 mM with one exception. No coenzyme was added to the solution containing apo-GAPDH ("Control"). Dehydrogenase activity of holo-GAPDH ("Control") was < 1% after 10 min. reaction time with ^{14}C-IAA.

c) In parentheses is the amount of dehydrogenase activity remaining following proteolysis and before addition of ^{14}C-IAA.

effect on the ability of chymotrypsin to inactivate rabbit muscle aldolase. The loss in dehydrogenase activity was facilitated by using undialysed crystalline enzyme or enzyme dialysed against 0.20 M $(NH_4)_2SO_4$(Tris-Cl;pH 8.0).

When the products of digestion with the four proteases are compared with respect to their ability to be alkylated with ^{14}C-iodoacetate in the presence of NAD^+, the differences listed in Table I are observed. The species formed by chymotrypsin modification of apo-GAPDH, which can still bind coenzyme (2), unambiguously displays facilitation of carboxymethylation that is attributed to coenzyme binding at a site in close proximity to the essential cysteine residue (7). The analogous modification by any of the other proteases, on the other hand, produces a species which reacts slowly with iodoacetate even in the presence of NAD^+, suggesting that these are less able (or unable) to bind NAD^+.

Comparison of the various species formed by proteolysis of the apo- and holoenzymes using SDS disc gel electrophoresis (Fig. 2) reveals that enzyme is little modified by chymotrypsin (Fig.2B) or trypsin (Fig.2D) when NAD^+ is present at high concentration. Under the same conditions both Pronase (Fig.2F) and subtilisin (Fig.2H) introduce several cleavages in GAPDH which are non-

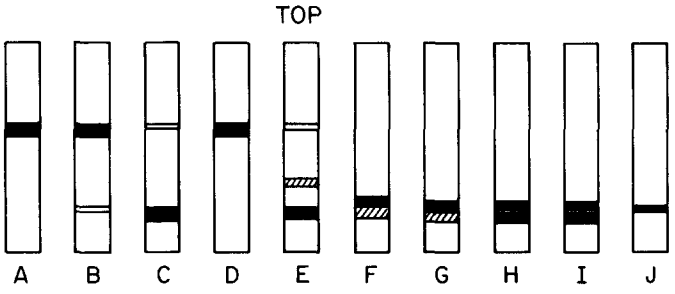

Figure 2. SDS Disc Gel Electrophoresis of GAPDH Products from Limited Proteolysis using Chymotrypsin and Trypsin. Conditions employed in modifying GAPDH may be found in Fig. 3. The tubes are identified according to the state of dehydrogenase (APO- or HOLO- enzyme) and the protease employed. GAPDH (monomer, mol.wt., 36,000), A; chymotrypsin-modified HOLO (B) and APO (C); trypsin-modified HOLO (D) and APO (E); Pronase-modified HOLO (F) and APO (G); subtilisin-modified HOLO(H) and APO (I); and lysozyme marker (mol.wt., 14,500).

lethal, i.e., the enzyme still retains its dehydrogenase activity, but produce low molecular weight species in SDS solution. All four proteases substantially degrade apo-GAPDH (Fig.2C,E,G, and I).

The correlation of change in enzymatic activity with coenzyme concentration is revealed in Fig. 3. Although the enzyme has four coenzyme binding sites, the major effect in terms of protection from proteolytic inactivation occurs when essentially one molecule of NAD^+ is present per molecule of the tetrameric enzyme.

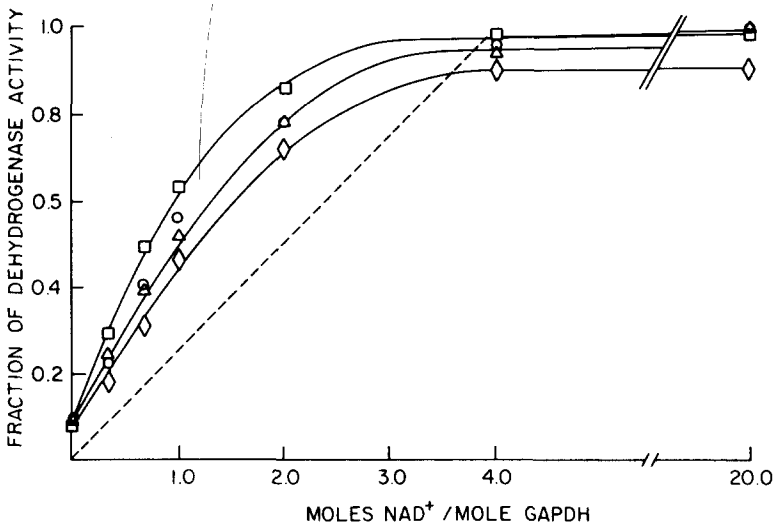

Figure 3. Effect of NAD^+ Concentration on the Proteolytic Inactivation of Rabbit Muscle GAPDH. Solutions (Tris-HCl, pH 8.0 with 0.20 M $(NH_4)_2SO_4$) of apo-GAPDH were incubated for 30 min. at 25°C with the appropriate amount of NAD^+. At the end of this period the protease (0.04%) was added and incubation was continued for 30 min. at 25°C. Digestion was terminated by adding NAD^+ (final conc. in all cases, 2.0 mM) and tosyl fluoride (0.30 mM) and by cooling the solutions at 4°C. The enzymatic activity of all samples was measured (6) before and after proteolysis; treatment of GAPDH with tosyl fluoride at even higher concentrations produced no deleterious effect. Symbols for protease employed: chymotrypsin (○), subtilisin (△), trypsin (□), and Pronase (◇). The dotted line represents a theoretical curve based on protease reacting with two distinct, non-interacting subunit conformers; one, "inactivatable" (no NAD^+ bound) and the other, "non-inactivatable" (NAD^+ bound).

Discussion - The effectiveness of NAD^+ at saturating concentrations in maintaining the functional integrity of rabbit muscle GAPDH in the presence of proteolytic enzymes has now been demonstrated with four proteolytic

enzymes, chymotrypsin, trypsin, subtilisin, and Pronase (Fig.1). It might be argued that the coenzyme when bound directly protects a limited number of susceptible peptide bonds from enzymatic hydrolysis. This proposition is supported to some extent by the observations that, with one exception (chymotrypsin modification), protease-modified apoenzyme will not sufficiently tightly bind coenzyme and display the rate enhancement effect during alkylation of the essential cysteine residue with iodoacetate. The pattern of fragments as determined by SDS disc gel electrophoresis demonstrates that, at least, for Pronase and subtilisin not all of the possible cleavage sites are protected when NAD^+ is bound; however, the resulting cleavages do not lead to a reduction in enzyme dehydrogenase activity. With trypsin a remarkable effect is obtained, namely bound coenzyme drastically reduces the rate of peptide bond hydrolysis at the normally "exposed" or minimally hydrophilic basic amino acid residues. Quite possibly hydrolysis at one (or a few) of these residues masked by bound NAD^+ so unhinges the protein tertiary structure that subsequent proteolysis follows easily. Use of a similar explanation for the results of chymotrypsin treatment (which also seems to leave intact the holoenzyme) leads to difficulties in explaining the exception noted earlier. Also the role played by ammonium sulfate in facilitating these proteolyses needs better defining. All in all, the results of studies discussed so far indicate that the differences in protease digestibility of the apo- and holoenzyme represent another manifestation of the substantial structural change that accompanies coenzyme binding (11,12,13).

However, the results at low NAD^+ concentration (Fig.3) can only be interpreted by assuming a global conformational change in protein quaternary structure upon the binding of coenzyme molecule, particularly the first, to the apoenzyme. Specifically when one equivalent of NAD^+ is present, only one-fourth of the binding sites should be occupied and directly protected; in actuality more than a half of the sites are protected from proteolytic

digestion. This estimate of the fraction of functioning active sites follows from the determination of enzymatic activity, provided these sites are inherently equivalent in catalytic ability. This is apparently the case with the conditions employed in this study (6).

These data may be viewed from another perspective. Because the enzyme so tightly binds (at least the first three molecules of) the coenzyme (4,5), Figure 3 may be considered (as a first approximation) to be a correlation between functions of state (ordinate) and saturation (abscissa). The symmetry model of Monod, Wyman and Changeux (MWC) (15) can be made to account for the observed curvilinear relationship. On the contrary the simplest sequential model of Koshland, Nemethy, and Filmer (KNF) (16) predicts a straight line relationship for this correlation. However, the behavior of coenzyme binding to the rabbit muscle enzyme, as originally described by Conway and Koshland (4), involves negative cooperative interactions between ligand binding sites that can only be rationalized by a more complex sequential mechanism. The data presented in this communication are consistent with a sequential model in which one subunit interacts either strongly with two of the remaining three subunits or less strongly with all three. Ligand binding to this subunit induces a conformation change in the interacting subunits to a "non-inactivatable" (or a "non-inactivatable"-like) form which has a decreased affinity for the ligand.

Clearly, however, these results <u>per se</u> do not distinguish between the MWC or complex KNF (or possible alternative) models. The ultimate model must certainly deal with changes in quaternary structure of the intact tetrameric enzyme, since the concentrations of protein (> 13 mg/ml) seem to exclude the possibility of association-dissociation phenomena for explaining the observed results (13). However, mention must be made of earlier dissociation studies carried out in ammonium sulfate solutions (0.15M) with lower enzyme concentrations (0.10 mg/ml) at 0°C (14). Additional evidence is being sought in order to provide a more rigorous establishment

of the mechanism for these ligand binding effects on limited proteolysis. Furthermore, preliminary experiments indicate that this same approach may prove useful in studying the corresponding coenzyme binding phenomena for the yeast enzyme, which have already been described (17) in terms of the MWC model.

References

1. Krimsky, I., and Racker, E., Biochemistry, 2, 512 (1963).
2. Cantau, B., Brunel, C., and Pudles, J., Biochim. Biophys. Acta, 167, 511 (1968).
3. McClintock, D.K., and Markus, G., J. Biol. Chem., 244, 36 (1969).
4. Conway, A., and Koshland, D.E., Jr., Biochemistry, 7, 4011 (1968).
5. de Vijlder, J.J.M., and Slater, E.C., Biochim. Biophys. Acta, 167, 23 (1968).
6. Fenselau, A., and Weigel, P., Biochim. Biophys. Acta, 198, 192 (1970).
7. Fenselau, A., J. Biol. Chem., 245, 1239 (1970).
8. Weber, K., and Osborn, M., J. Biol. Chem., 244, 4406 (1969)
9. Fox, J.B., Jr., and Dandliker, W.B., J. Biol. Chem., 221, 1005 (1956).
10. Nomoto, M., Narahashi, Y., and Murakami, M., J. Biochem. (Japan), 48, 593 (1960).
11. Listowsky, I., Furfine, C.S., Betheil, J.J., and Englard, S., J. Biol. Chem., 240, 4253 (1965).
12. de Vijlder, J.J.M., and Harmsen, B.J.M., Biochim. Biophys. Acta, 178, 434 (1969).
13. Hoagland, V.D., Jr., and Teller, D.C., Biochemistry, 8, 594 (1969).
14. Constantinides, S.M., and Deal, W.C., Jr., J. Biol. Chem., 245, 246 (1970).
15. Monod, J., Wyman, J., and Changeux, J.P., J. Mol. Biol., 12, 88 (1965)
16. Koshland, D.E., Jr., Nemethy, G., and Filmer, D., Biochemistry, 5, 365 (1966).
17. Kirschner, K., Eigen, M., Bittman, and Voigt, B., Proc. Nat. Acad. Sci. (USA), 56, 1661 (1966).