BBA 66051

STRUCTURE–FUNCTION STUDIES ON GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

II. THE EFFECTS OF S-CARBOXYMETHYLATION ON ENZYMATIC ACTIVITY

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(Received September 8th, 1969)

SUMMARY

Enzymatic activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was correlated with the number of active site cysteine residues alkylated by iodoacetate under various conditions. A one-to-one correspondence between loss of dehydrogenase activity and incorporation of [^{14}C]carboxymethyl groups (up to 3.5 groups per enzyme molecule) was observed at pH 8.0 and 7.0 in the presence of glyceraldehyde 3-phosphate, arsenate or NAD^+. Comparable studies using enzyme completely or partially acylated with 3-phosphoglyceryl groups provided identical results. The conclusion based on these observations is that the four alkylatable cysteine residues in the tetrameric protein are intrinsically equivalent in the sense that each can function in the enzymatic reaction.

INTRODUCTION

An understanding of the role of subunits in the functioning of multichain enzymes stands as an intriguing challenge among present-day problems of enzymology. In some cases, such as aspartate transcarbamylase[1], a specific function (catalytic or regulatory) can be assigned to each set of structurally different subunits. However, only with respect to hemoglobin, a non-enzyme, do we have some insight into the dynamics of the interaction between the polypeptide chains in an oligomeric protein[2,3]. Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD^+-oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle is composed of four identical or near-identical subunits[4]. Studies on the hydrodynamic properties[5–7] and crystal structure[8] indicate that the dimer of these subunits may be a fundamental structural element. Non-identity of the subunits in the tetramer in a functional sense has been suggested on the basis of a number of physicochemical investigations, for example those of CONWAY AND KOSHLAND[9], MALHOTRA AND BERNHARD[10], and in the references cited by these authors. Our interest in this matter

was initially in developing methods for separating very similar polypeptide chains in which a primary structural difference due to a chemical modification could be effected by utilizing a functional difference between the sets of subunits. However, our efforts at first detecting functional differences under the physiological conditions employed in earlier reports were unsuccessful. That is to say, all four active sites carboxymethylated by iodoacetate in non-denaturing media are fully essential to the catalytic performance of the enzyme. Also substrate protection and coenzyme activation of the essential cysteine residues do not lead to a disproportionate change in activity as the alkylation reaction proceeds.

EXPERIMENTAL PROCEDURE

Enzyme preparation

The rabbit muscle enzyme employed in this investigation was purchased from Boehringer Mannheim Corp. (specific activity, 60–70 I.U.). Electrophoresis on cellulose acetate strips indicated the commercial preparation was homogeneous, containing only one protein component (using Ponceau S stain) which also displayed dehydrogenase activity. The dehydrogenase staining method of LEBHERZ AND RUTTER[11] was modified by replacing the glyceraldehyde 3-phosphate generating system with D-glyceraldehyde (20 mg/ml). Polyacrylamide (4.9%) gel electrophoresis in a Tris–glycine buffer (pH 8.3) did reveal the presence of a second minor component. Charcoal treatment was carried out in a batchwise manner[12] and provided apoenzyme with $A_{280\text{ nm}}:A_{260\text{ nm}}$ of > 1.8 and with $> 90\%$ of the activity of the holoenzyme. Extinction coefficients of FOX AND DANDLIKER[13] were used to determine protein concentrations for both apo- and holoenzyme.

Radioisotopic labeling

Iodo[1-^{14}C]acetate (Volk Radiochemical Co.) had a specific activity of $5.1 \cdot 10^5$

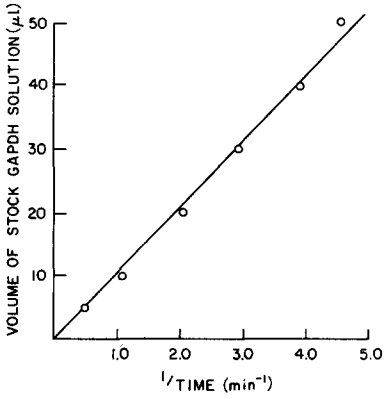


Fig. 1. Relationship between glyceraldehyde-3-phosphate dehydrogenase concentration and the time required to reduce a given amount of NAD+. Conditions: the assay solution (stated in order of addition) contained sodium pyrophosphate buffer (0.05 M, pH 8.5) with 1 mM EDTA, sodium arsenate (13 mM), NAD+ (170 μM), glyceraldehyde-3-phosphate dehydrogenase (stock solution, 10 μM), and glyceraldehyde 3-phosphate (170 μM). Final volume: 3.0 ml. In this case the total amount of reduced NAD+ per cuvet was 0.94 μmole. See EXPERIMENTAL PROCEDURE (*Enzymatic assays*) for other details of these measurements.

disint./min per μmole, as determined by conversion to glycine and measuring concentration on a Beckman Spinco model 120B amino acid analyzer and disint./min on a Packard Tricarb scintillation counter. The matrix method using glass fiber discs for following incorporation of radiocarbon into the protein has been described elsewhere[14]. All other chemicals were obtained from Sigma Chemical Co.

Enzymatic assays

The method for following changes in enzyme dehydrogenase activity is essentially the standard procedure[15]; however, data collecting and processing were modified for this study. All assays were carried out using a Gilford model 222 spectrophotometer with thermostated cuvet holder (maintained at 25.0°) and a Sargent SRL recorder with chart speeds of 1 and 5 inches/min. A stock assay solution (see Fig. 1 for details) was prepared in advance and kept at 25.0°. An aliquot of the enzyme solution to be assayed was incubated at room temperature for 2 min in a solution (at pH 8.5) containing sodium pyrophosphate (0.05 M), EDTA (1 mM) and dithiothreitol (2 mM). (Aliquots from the reaction run in arsenate medium (0.1 M) were allowed to stand 12 min in the incubation solution before assaying for dehydrogenase activity.) Following addition of enzyme to the assay solution and nulling the cuvet at 340 nm, glyceraldehyde 3-phosphate was introduced using a plumper (Calbiochem) at the same time as the recording was started. The reference assay (measured at zero time and various subsequent times on a solution of the enzyme containing all additions except iodoacetate) was recorded with a chart speed of 5 inches/min. At various times during the course of the reaction with iodoacetate, the solution was assayed in an analogous manner. Assays in all cases were reproducible to within 5%. In order to measure relative activities, the times required to develop a specific absorbance (at 340 nm) were compared for control and iodoacetate-treated enzyme solutions. In Fig. 1 may be seen the correlation between concentration of enzyme used in the assay and the reciprocal of the time required to produce a given amount of reduced coenzyme (in this case, 0.94 μmole). The range of activities measured in this instance corresponds to that being measured in the inactivation studies.

RESULTS

The alkylation reaction of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was allowed to proceed with iodoacetate under a variety of conditions, which have been summarized in Table I. The reactivity of the 3.5 alkylatable sulfhydryl groups, as expressed by a $t_{\frac{1}{2}}$ value, is dependent on hydrogen ion concentration (greater at higher pH values due to increased nucleophilicity of the sulfhydryl groups[16]) and the presence of NAD^+, glyceraldehyde 3-phosphate or sodium arsenate. Enhancement of the reaction due to the presence of NAD^+ has been observed previously[17,18] and seems to be best explained in terms of a specific ionic interaction between coenzyme and inhibitor[14]. The expected protection by glyceraldehyde 3-phosphate (by formation of a hemithioacetal with the enzyme sulfhydryl group)[17,19] was also apparent. When the fraction of dehydrogenase activity was measured on the same solutions undergoing reaction with iodoacetate, a linear relationship with the fraction of alkylatable (under these mild, non-denaturing conditions) cysteine residues in the enzyme is noted in all cases (Figs. 2 and 3). Such a straight line is expected on the

TABLE I

EFFECTS OF VARIOUS SUBSTANCES ON THE TIMES REQUIRED FOR CARBOXYMETHYLATING ONE-HALF OF THE SH GROUPS AT THE ACTIVE SITE OF RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

All reaction solutions contained 49 μM glyceraldehyde-3-phosphate dehydrogenase and 230 μM iodo[1-^{14}C]acetate and were maintained at 25°. The concentrations of the various additions are listed in the table. The pH of each solution was adjusted to the appropriate value before mixing with the enzyme solution (also at the same pH). 0.5 min after withdrawing the 50-μl aliquot used in determining the ^{14}C content, another aliquot was removed and transferred to the incubation solution. The times, $t_{1/2}$, required for alkylating half of the final number of cysteine residues are included in the table. Complete inactivation corresponds to the incorporation of 3.5 $\pm$ 0.2 carboxymethyl groups.

Addition (concn.)	$t_{1/2}$ *(min)*
pH 8.0 (undialysed apoenzyme)	
No addition	9
NAD^+ (2.0 mM)	1.5
Glyceraldehyde 3-phosphate (2.9 mM)	28
Glyceraldehyde 3-phosphate (9.9 mM)	60
pH 7.0 (dialysed apoenzyme)	
No addition	10
NAD^+ (2.0 mM)	0.5
Na_2HAsO_4 (0.10 M)	165
Glyceraldehyde 3-phosphate (2.9 mM)	225

basis of two assumptions: the four active sites are (a) identical and (b) non-interacting. In the sense used here "identical" is defined operationally in terms of the capability of a given site in effecting enzymatic reaction to the same extent as any other site; "non-interacting" means that carboxymethylation of one site does not affect catalytic functioning at any other site.

Protection of the active site cysteine residue was accomplished in another fashion by synthesizing the thioester enzyme intermediate according to the procedure detailed by KRIMSKY AND RACKER[20]. In Table II may be seen the results from alkylating with iodoacetate the fully and partially acylated enzyme, deacylating by arsenolysis, and assaying the iodoacetate-treated enzyme for dehydrogenase activity. Here, too, full protection of enzyme activity is related to full protection of the alkylatable cysteine residues from iodoacetate (line B). The same correspondence in loss of activity and gain in [^{14}C]carboxymethyl groups is found when protection by the 3-phosphoglyceryl group is deliberately made incomplete (line C).

DISCUSSION

Correlations of quaternary structure with enzymatic function are in a preliminary stage for glyceraldehyde-3-phosphate dehydrogenase. The existence in solution of the dimeric species has recently been shown for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase under a variety of conditions, *e.g.*, low protein

TABLE II

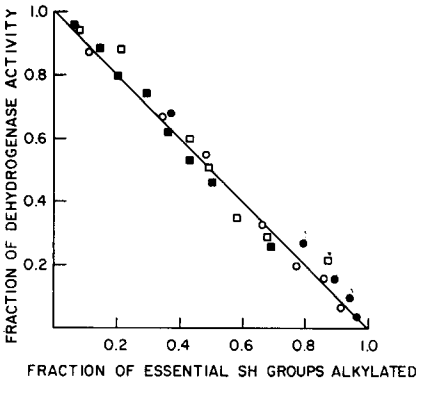
CORRELATION OF CHANGE IN DEHYDROGENASE ACTIVITY AND EXTENT OF ^{14}C CARBOXYMETHYLATION IN RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN WHICH THE ACTIVE SITE SH GROUPS HAVE BEEN ACYLATED UNDER PHYSIOLOGICAL CONDITIONS

Conditions: additions were made to the holoenzyme in order stated to give 0.25 ml as the final volume. (A) Buffer (Tris-HCl, 0.05 M), NAD^+ (2.8 mM), sodium pyruvate (2.8 mM), glyceraldehyde-3-phosphate dehydrogenase (51 μM), and rabbit muscle lactate dehydrogenase (40 μg/ml). Final pH 7.5. The solution was assayed for dehydrogenase activity, and then iodo[^{14}C]acetate (0.36 mM) was added. After 15 min of reaction time at room temperature, a 50-μl portion was removed to a glass fiber disc (in order to process the sample for scintillation counting) and, immediately following this, an aliquot was withdrawn for determining dehydrogenase activity. (B) Conditions were identical to (A) except that glyceraldehyde 3-phosphate (5.3 mM) was added after sodium pyruvate. Following a pH adjustment, the enzymes were added. Final pH 7.8. After 90 min at 0° the solution was assayed, iodo[^{14}C]acetate (0.36 mM) was added, and, following 15 min of reaction time, the solution was treated as in (A). (C) Conditions were identical to (B) except for changes in concentration of NAD^+ (0.12 mM) and glyceraldehyde 3-phosphate (0.53 mM). Final pH 7.3. After 5 min at 0°, the reaction solution was treated as in (B).

	Activity (%)	*Alkylation* (%)
(A) No protection	2	98
(B) Full protection	95	4.3
(C) Partial protection	74	28

concentrations at neutral pH with either no additions[5] or ATP present[6] or moderate protein concentrations in an aqueous medium of high ionic strength containing dimethylsulfoxide[7]. The asymmetric unit in the crystals of lobster muscle glyceraldehyde-3-phosphate dehydrogenase is the dimer and not the monomer, as revealed by the X-ray diffraction studies of WATSON AND BANASZAK[8]. Optical rotatory dispersion[21,22] and circular dichroism[23] studies as well as viscosity[24] and sedimentation velocity[5] measurements clearly indicate that the greatest structural change induced by coenzyme binding to the rabbit muscle enzyme occurs upon binding the first molecule of NAD^+. The first molecule of NAD^+ has also been shown to bind to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase considerably more rapidly than subsequently bound coenzyme molecules[25]. Interaction between the four coenzyme binding sites in the enzyme from the same source has been established by CONWAY AND KOSHLAND[9] in their investigations on negative cooperativity as a general phenomenon of oligomeric proteins. Differential reactivity of the active site cysteine residues, *i.e.*, the preferential acylation of one or two of the four residues, has been demonstrated using various reagents, β-(2-furyl)acrylolyl phosphate[10], acetyl phosphate[26], and several highly branched acyl phosphates[27]. For each of these examples the effect is manifested in the presence of optimal amounts of NAD^+. CHANCE AND PARK[26] concluded from spectroscopic measurements on the formation of yeast enzyme–coenzyme complex and, at the same time, on the formation of reduced coenzyme from the oxidation of substrate that only a small fraction (about 25%) of the total complex is actually involved in the oxidation reaction. Thus, a reasonable conclusion based on present information is that functional non-equivalence of the subunits in the tetrameric protein can be induced by binding NAD^+.

Furthermore, differential protection of the cysteine residues has been reported



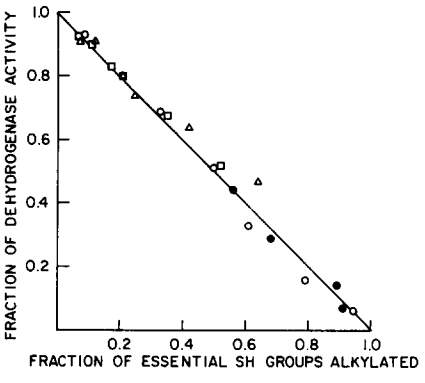


Fig. 2. Correlation of the amount of dehydrogenase activity with the amount of carboxymethylation at pH 8.0 of the active site cysteine residues of undialysed glyceraldehyde-3-phosphate dehydrogenase. The straight line corresponds to the theoretical curve based on reaction of iodoacetate with four identical and independent active site sulfhydryl groups. Conditions: undialysed apo-glyceraldehyde-3-phosphate dehydrogenase (49 μM) at pH 8.0 (Tris–HCl buffer, 0.05 M) was allowed to react with iodo[1-^{14}C]acetate (230 μM) in the presence of various substances: ○, no additions; ●, NAD^+ (2.0 mM); □, glyceraldehyde 3-phosphate (2.0 mM); ■, glyceraldehyde 3-phosphate (9.9 mM). See Table I and text for additional details.

Fig. 3. Correlation of the amount of dehydrogenase activity with the amount of carboxymethylation at pH 7.0 of the active site cysteine residues of dialysed glyceraldehyde-3-phosphate dehydrogenase. Conditions differed from those presented in Fig. 2 in that apo-glyceraldehyde-3-phosphate dehydrogenase was first dialysed against Tris–HCl buffer (pH 7.0) and then (at a concentration of 49 μM) was allowed to react at this pH with iodo[1-^{14}C]acetate (230 μM) in the presence of various substances: ○, no additions; ●, NAD^+ (2 mM); □, glyceraldehyde 3-phosphate (2.9 mM); △, Na_2HAsO_4 (0.10 M). See Table I for additional details.

by SEGAL AND BOYER[17] when the reaction with iodoacetate is carried out in the presence of glyceraldehyde 3-phosphate. The results or our studies, particularly those carried out with glyceraldehyde 3-phosphate or arsenate present or with no additions, reveal that any loss in enzymatic activity is equalled by a corresponding loss in the four (or as actually measured, 3.5) "essential" sulfhydryl groups due to carboxymethylation. This conclusion is at variance with that of SEGAL AND BOYER[17] who reported only two of the four sulfhydryl groups usually alkylated with iodoacetate are necessary for enzyme activity. Employing conditions comparable to those of SEGAL AND BOYER and analytical methods described above, we were unable to observe any evidence supporting the earlier conclusion. Determinations of the effects of other factors (such as pH, NAD^+ and arsenate) by following, over a period of time for a given reaction solution, the changes in activity and amount of [^{14}C]carboxymethyl groups introduced into the homogeneous preparation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase provide further support for our conclusion. Comparable results were obtained by complete and partial protection of the active site cysteine residues from iodoacetate attack using the 3-phosphoglyceryl group covalently bound to the enzyme. Furthermore, recent analogous studies also report that loss in glyceraldehyde-3-phosphate dehydrogenase activity is linearly dependent on the amount of tetrathionate or *p*-mercuribenzoate and of iodoacetate that has combined with rabbit muscle[28] and lobster tail muscle[29] enzymes, respectively.

Complete inactivation in these studies required 3.7–3.8 moles of covalently bound inhibitor per mole of enzyme. Kinetic data obtained on rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was interpreted by FURFINE AND VELICK[30] in terms of an enzyme molecule composed of multiple (3.1) NAD^+ binding sites which are all equivalent but become modified under reaction conditions.

Therefore, it seems reasonable to state that each of the four subunits in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is fully able to catalyze the oxidation of glyceraldehyde 3-phosphate, *i.e.*, the subunits are inherently equivalent in enzymic functioning. However, some agents, certainly NAD^+, several acylating reagents and possibly ATP, under specified conditions can perturb this state of functional equivalence and can produce an observable functional non-equivalence among the subunits. Questions relating to the mechanism for these events at a submolecular level and to their physiological significance will figure prominently in future efforts at correlating structure with function for glyceraldehyde-3-phosphate dehydrogenase.

ACKNOWLEDGEMENTS

This investigation was supported by a grant GB-7326 from the National Science Foundation and an institutional research grant from the American Cancer Society. P. W. gratefully acknowledges support as a Public Health Service predoctoral trainee by a National Institutes of Health grant (GM-00181).

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