BBA 66031

STRUCTURAL STUDIES ON THE ACTIVE CENTER OF α-GLYCEROLPHOSPHATE DEHYDROGENASE

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(Received August 11th, 1969)

SUMMARY

1. In an attempt to clarify the structure of the active center of α-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), the behavior of the kinetics parameters of the enzyme as a function of pH was examined.

2. The values of 6.61 found for the pK_1 of the free enzyme and 6.72 for the pK_1 of the enzyme–substrate complex suggested the presence of at least one histidyl residue at the active center.

3. Photo-oxidation of the enzyme in the presence of Rose Bengal as sensitizer inactivated the enzyme. This inactivation was dependent on the destruction of a group or groups having a pK_a of 7.3.

4. Chemical modification of the enzyme with diazo-1-H-tetrazole, under conditions where neither bisazohistidine nor monoazo- or bisazotyrosine could be detected, showed that the conversion of 1.07 histidine to the monoazo product led to a 50% inactivation. When 1.77 monoazohistidine residues had been formed, the level of inactivation was 94%. Complete inactivation was attained when 2.14 histidines had been converted to the monoazoderivative.

5. The results reported here strongly suggest that two histidine residues at the active center of the α-glycerolphosphate dehydrogenase are critical for the activity of this enzyme.

INTRODUCTION

The cytoplasmic NAD-linked L-glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8), which catalyzes the interconversion of dihydroxyacetone phosphate and glycerol 3-phosphate, has been the subject of many biochemical investigations. These studies have been mainly focused on its physicochemical properties[1–4], coenzyme binding[5–8] and kinetic behavior[9–16].

Since this enzyme has an intramitochondrial complement, glycerolphosphate oxidase (EC 1.1.2.1), which catalyzes the same reaction using a flavin as coenzyme[17–20]

a comparative study of the structure of the active center and of the reaction mechanism of these enzymes would be of great value for a better understanding of enzyme action.

To our knowledge, the only studies attempting to probe the structure of the active center of glycerolphosphate dehydrogenase have dealt with the function of the sulfhydryl groups[21]. From these studies and those on the fluorescent behavior of the enzyme inhibited with *p*-chloromercuribenzoate[22], it seems established that the sulfhydryl groups of the enzyme do not participate directly in the reaction, but that they do play an important role in the maintenance of a conformational state of the protein which is critical for enzymatic activity.

In this paper we report the results of a study of the kinetics and chemical modifications of α-glycerol-3-phosphate dehydrogenase, which strongly suggest the importance of histidyl groups for the enzyme's catalytic action.

MATERIALS

L-α-Glycerolphosphate dehydrogenase from rabbit skeletal muscle (specific activity, 80–100 units/mg at pH 7.5 and 25°) was purchased either from Boehringer-Mannheim or from Sigma Chemical Co., St. Louis. Dihydroxyacetone-phosphate as the dimethylketal dicyclohexylammonium salt, NAD^+, NADH (disodium salt), triethanolamine–HCl, and Tris were obtained from Boehringer-Mannheim. DL-α-Glycerolphosphate, Grade X, was purchased from Sigma Chemical Co., St. Louis. Rose Bengal 2B (Edward Gurr) was purified as described by BRAND *et al.*[23]. 5-Amino-1-H-tetrazole monohydrate, m.p. 204° (decomp.), was obtained from Aldrich Chemical Co., Milwaukee, and used without further modification. Twice-distilled, deionized water was used throughout. pH measurements were made with a Beckman Research pH meter equipped with a glass electrode.

METHODS

Enzyme activity in the forward reaction (dihydroxyacetonephosphate → α-glycerolphosphate) was measured by changes in absorbance of NADH at 334 nm as a function of time (Eppendorf photometer) as described previously[9]. For the reverse reaction (α-glycerolphosphate → dihydroxyacetonephosphate), activity was measured at 334 nm in Tris buffer 50 mM (pH 8.3) at a final concentration of enzyme of 4.5 nM, NAD^+ 20 mM and L-α-glycerolphosphate 25 mM at 25°. Protein concentration was determined by the method of WADDELL[24], as modified by MURPHY AND KIES[25]. Enzyme concentrations were calculated on the basis of a molecular weight of 70 000 (refs. 2, 3 and 5).

Enzymatic reaction kinetics were studied for the forward reaction. For each determination of K_m and v_{max}, eight substrate concentrations were used ranging from 1.0 μM to 50 μM ($[S] < K_m$). Preliminary double reciprocal plots ($1/v$ against $1/[S]$) were made visually. When these plots appeared to be linear, the data were subjected to computer analysis by the method developed by WILKINSON[26].

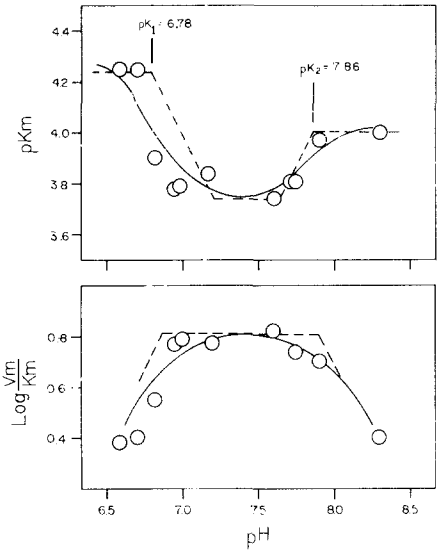
Values of K_m and v_{max}, determined as a function of pH, were plotted in a logarithmic scale and interpreted as suggested by DIXON[27]. Each K_m and v_{max} de-

termination was done on the same day, with the same enzyme and substrate dilution. Substrate concentration was calculated from enzymatic analysis.

Photo-oxidation of the enzyme was carried out at 4°, in triethanolamine buffer, 0.1 M, at the appropriate pH, under gentle agitation, with Rose Bengal as sensitizer. A 500-W photoflood lamp was used at a distance of 10 cm from the probe.

5-Diazo-1-H-tetrazole was prepared by diazotization of 5-amino-1-H-tetrazole as described by HORINISHI *et al.*[28]. The coupling reaction was carried out in 1 M $KHCO_3$ (pH 8.8) at 20°. The molar absorbances (ε) reported by SOKOLOVSKY *et al.*[29] were used for calculations. For the time-dependence studies of 5-diazo-1-H-tetrazole coupling and enzyme activity, the coupling reaction was quenched by diluting an aliquot with 0.1 M Tris buffer (pH 7.5) in order to arrest any further modification of the enzyme[30].

For the absorption measurements, 5-diazo-1-H-tetrazole was added simultaneously to the probe and to a buffer control, and changes in absorption with time were recorded on a dual-beam spectrophotometer at 360 nm (Bausch and Lomb, Spectronic 505).



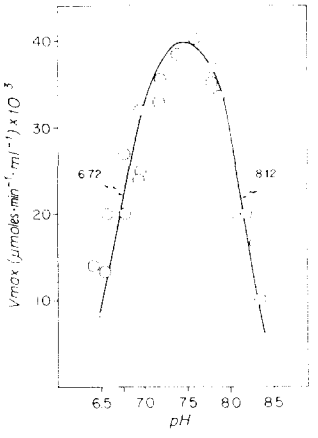


Fig. 1. pK_m and log (v_{max}/K_m) values as a function of pH, for the reduction of dihydroxyacetonephosphate catalyzed by α-glycerolphosphate dehydrogenase. Data were obtained and analyzed as described in METHODS, each circle represents the mean value of four experiments.

Fig. 2. Maximal velocities for the reaction of α-glycerolphosphate dehydrogenase as a function of pH. The values on the ordinate are enzyme units/ml of an original solution of arbitrarily fixed concentration. Symbols indicate values obtained by computer analysis of velocity measurements as a function of substrate concentration. Each point represents the mean value of four experiments. The solid line is the theoretical bell-shaped curve calculated as the best fit to the experimental points (see text).

RESULTS AND DISCUSSION

The dependence of K_m on pH for the enzymatic reduction of dihydroxyacetonephosphate is shown in Fig. 1, in which pK_m and log (v_{max}/K_m) are plotted as a function of pH. As can be seen from this figure these quantities depend on the state of ionization

of two groups having values of pK_a of 6.78 and 7.86. The pK_1 value of 6.78 obtained for the free enzyme agrees well with those generally found in protein molecules for imidazol groups[31] and suggests the participation of a histidine residue at the active center.

Direct titration of the substrate, dihydroxyacetonephosphate, performed under the same conditions as those employed for the activity measurements, gave a value of 7.4 for the second ionization constant of the phosphate group. This value suggests that a phosphate ionization of the substrate is not responsible for the pK_1 obtained. The value of 7.8 obtained for pK_2 could also tentatively be assigned to another histidine residue of the free enzyme, although a terminal amino group could not be eliminated on the basis of the kinetic studies alone[31].

The dependence of v_{max} on pH for the reduction of dihydroxyacetonephosphate is shown in Fig. 2. From this curve, a pK_{ES_1} of 6.72 and a pK_{ES_2} of 8.12 were obtained. Analysis of the date in terms of the equation:

$$v = \frac{V_{opt}}{1 + \frac{[H_+]}{K_{ES1}} + \frac{K_{ES2}}{[H^+]}}$$

demonstrated fairly good agreement with the bell-shaped curve predicted by Eqn. 1, in which V_{opt} is the maximum velocity at the pH optimum, v is the velocity at substrate saturation for other values of pH, and K_{ES_1} and K_{ES_2} are the first and second dissociation constants for the enzyme–substrate complex.

The pK_1 of 6.72 obtained in this experiment also suggests that at least one histidyl residue is present at the active center of the enzyme. Although the value of 8.12 for the pK_2 of the enzyme–substrate complex is difficult to assign to any specific amino acid residue, another histidine group would not be excluded. The pK values for free enzyme and enzyme–substrate complex which determine the kinetic behavior are summarized in Table I.

TABLE I

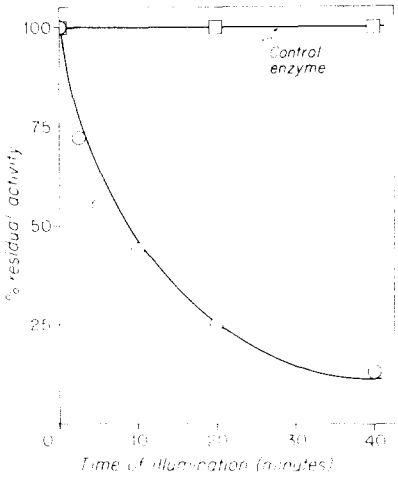
IONIZATION CONSTANTS FOR THE REDUCTION OF DIHYDROXYACETONEPHOSPHATE BY α-GLYCEROLPHOSPHATE DEHYDROGENASE

State of the enzyme	pK_1	pK_2
Free enzyme*	6.78	7.86
Enzyme–substrate complex**	6.72	8.12

* From pK_m and log (v_{max}/K_m) *versus* pH plots.
** From computer analysis of v_{max} *versus* pH (see text).

In order to obtain more direct information concerning the role of histidine residues at the active center of α-glycerolphosphate dehydrogenase, photo-oxidation of the enzyme with Rose Bengal as sensitizer and chemical modification experiments with diazo-1-H-tetrazole were performed. As shown in Fig. 3, photo-oxidation of the α-glycerolphosphate dehydrogenase with Rose Bengal as sensitizer inactivates the enzyme rapidly.

This inactivation was found to be pH dependent, and as can be seen in Fig. 4,



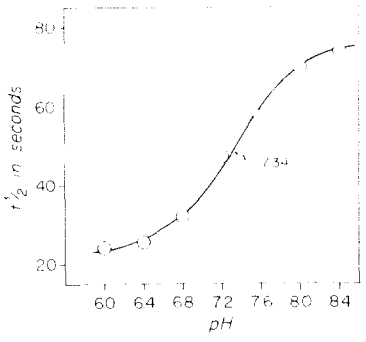


Fig. 3. Inactivation of rabbit-muscle α-glycerolphosphate dehydrogenase during photo-oxidation in the presence of Rose Bengal. The experiment was carried out at pH 6.4, as described in METHODS; enzyme concentration was 0.28 μM; Rose Bengal was 0.1 μM. Residual activity was measured in triethanolamine–HCl buffer, 50 mM, pH 7.5 (25°) at a final enzyme concentration of 4.6 nM. Controls were done either by illuminating the enzyme in the absence of Rose Bengal or with Rose Bengal in the dark.

Fig. 4. pH-dependence of the photo-oxidation of α-glycerolphosphate dehydrogenase using Rose Bengal as sensitizer. Triethanolamine buffer, 0.1 M, was used throughout. Other conditions as in Fig. 3.

the inactivation depends on the protonated form of a group or groups with a pK_a of 7.3.

It is well-known[32] that dye-sensitized photo-oxidation of proteins may modify not only histidine but also methionine, tryptophan and tyrosine residues. However, only the photo-oxidation of histidine has been found to be sensitive to pH changes[33]. Furthermore, since Rose Bengal is an anionic photoactive dye, the fact that the histidine residue must be in the protonated form for oxidation corroborates the work of SLUYTERMAN[34], who established that a short-lived complex is formed between the activated dye and its substrate prior to the transfer of oxygen. The pK_a of 7.3, found for the photo-oxidation inactivation, substantiates the suggestion that one or more critical histidyl residues form part of the active center of α-glycerolphosphate dehydrogenase.

A more direct indication of the participation of histidine at the active center of the enzyme comes from studies of the chemical modification of the enzyme with diazo-1-H-tetrazole.

The coupling of the enzyme with diazo-1-H-tetrazole in 1 M $KHCO_3$ (pH 8.8) results in a loss of enzymatic activity. This decrease in activity is dependent on the molar excess of 5-diazo-1-H-tetrazole, giving a 96% inactivation with a 200-fold molar excess of 5-diazo-1-H-tetrazole over enzyme (Fig. 5). As is well-known[2,4], α-glycerolphosphate dehydrogenase contains about 14 histidine moles per mole of enzyme (70 000 g); therefore, a 200-fold molar excess of 5-diazo-1-H-tetrazole represents only a 14-fold excess over the histidine content of the enzyme and a 25-fold excess over the tyrosine content (8 residues per mole of 70 000 g). Under these con-

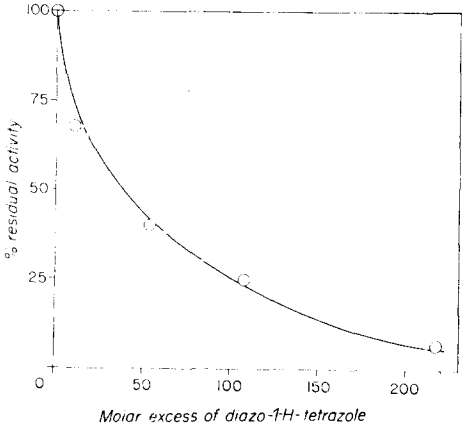


Fig. 5. Effect of variation of the molar excess of diazo-1-H-tetrazole on the enzymatic activity of α-glycerolphosphate dehydrogenase. The coupling reaction was quenched after 10 min by diluting an aliquot with 0.1 M Tris buffer (pH 7.5).

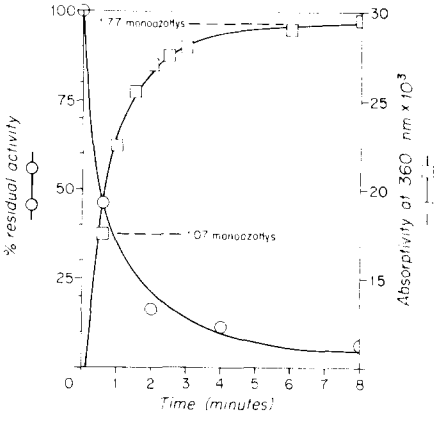


Fig. 6. Correlation of the variations in absorption at 360 nm with the loss of enzymatic activity of α-glycerolphosphate dehydrogenase during coupling with a 200-fold excess of diazo-1-H-tetrazole. Changes in absorption were recorded as described in METHODS. A cell with a 2.0-cm light path was used in these experiments. The final enzyme concentration was $7.10 \cdot 10^{-7}$ M. An $\varepsilon_{(360\ nm)} = 11.5 \cdot 10^3$ (ref. 29) for the monoazohistidine was used for calculations.

ditions, neither bisazohistidine nor monoazo- or bisazotyrosine could be detected by spectrophotometric analysis at 480 and 550 nm after 20 min coupling with 5-diazo-1-H-tetrazole in 1 M $KHCO_3$ (pH 8.8). However, if the coupling reaction is followed at 360 nm, where monoazohistidine absorbs strongly, a very rapid increase of absorption with time is observed. Fig. 6 exhibits the decrease in enzymatic activity with time during the coupling reaction with 5-diazo-1-H-tetrazole and the increase in absorption at 360 nm observed concomitantly. By the use of a molar absorption coefficient of $11.5 \cdot 10^3$ for the monoazohistidine compound[29], it can be calculated that conversion of 1.77 histidine residues to the monoazo product results in a level of inactivation of 94%. A 53% inactivation is obtained when 1.07 histidine residues have been transformed to the monoazo product. The effect of 5-diazo-1-H-tetrazole coupling on the reverse reaction was quite similar to that on the forward reaction, *i.e.* 61% inactivation is attained when 1.2 monoazohistidine groups have been formed and the formation of 1.63 monoazohistidine groups leads to an 83% inactivation. Complete inactivation was reached after 10 min coupling, when 2.14 histidines were converted to the monoazo derivative.

The combined kinetic and chemical modification data reported here strongly suggest that in the active center of α-glycerolphosphate dehydrogenase there are two histidine residues of about equal reactivity towards the diazo reagent, at least one of which is essential for enzymatic activity.

ACKNOWLEDGMENTS

The authors are deeply indebted to Dr. Eugene H. Cordes and Dr. Karl Gaede for their invaluable observations and suggestions.

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