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*Leuconostoc mesenteroides*에서 分離한 Glucose-6-phosphate Dehydrogenase의 特性

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Characteristics of Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*

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요 약. 저자들은 Cibacron Blue F3G-A Separose 컬럼 어피니티크로마토그래피에 의하여GIncose-6-phosphate dehydrogenase를 *Leuconostoc mesenteroides*로부터 순수 분리한 바 있다. 이 효소를 사용하여 효소 특성을 조사한 결과 분자량은 Sephadex G-200 컬럼에 의해 112,000이었으며 최적온도는 50°C, 활성화에너지는 8.36 kcal/mole 불활성화에너지는 −58.2 kcal/mole로 나타났다. $NADP^+$를 조효소로 사용하였을때 최적 pH 7.8에서 K_{G6P}: 76.9 μM, αK_{NADP}: 7.46 μM, αK_{NNADP}: 7.14 μM 이었으며 같은 조건에서 NAD^+를 조효소로 사용하였을때 K_{G6P}: 53.65 μM, K_{NAD}: 115.2 μM αK_{NAD}: 707.2 μM이었다. 따라서 $NADP^+$ 및 NAD^+를 조효소로 사용한 경우에 있어서 α 값은 각각 1과 6으로 나타났다. pH 변화에 따른 반응속도상수의 변화에 의하면 NAD^+를 조효소로 하였을때 최적 pH는 7.8 이었고 *pKa* 가 7.2인 활성기와 *pKb*가 9.0~9.6인 활성기가 효소와 기질의 상호작용에 관여함을 알았다. 이중 *pKa* 7.2인 활성기를 밝히기 위하여 효소를 광산화와 carboxymethylation을 시킨결과 histidine의 imidazole 기임을 알수 있었다.

ABSTRACT. Glucose 6-phosphate dehydrogenase of *Leuconostoc mesenteroides* which was purifid by an affinity chromatography was studied on the characterization, kinetics and chemical modification.

The apparent molecular weight of the enzyme was 112,000 by the gel filtration method of Sephadex G-200 column. The optimum temperature of NAD^+-linked reation was 50 °C and the activation energy and the heat of inactivation were 8.36 kcal/mole and −58.2kcal/mole, respectively.

The steady state kinetic study showed K_{G6P}, K_{NADP}, and αK_{NADP} to be 76.9 μM, 7.46 μM, and 7.14 μM, respectively, and K_{G6P}, K_{NAD}, and αK_{NAD} to be 53.7 μM, 115.2 μM and 702.2 μM for the NAD^+-linked reaction at pH 7.8, optimum pH.

The pH dependent kinetic constants suggested that the two ionizing groups whose *pKa* is 7.2 and *pKb* is 9.0~9.6 were involved in the enzyme-substrate interaction. Evidence by photooxidation and carboxymethylation of the enzyme suggested that the imidazole group of histidine with *pKa* group may participate in the catalytic site.

INTRODUCTION

Glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) catalyzes the oxidation of G6P to 6-phosphogluconate in the presence of NAD^+. This enzyme is widely distributed in plant, mammalian systems[2~4], and microorganisms[5~11]. Most of G6PDH, for example, that in yeast, cannot use NAD^+ as a cofactor[5]. The enzyme from *L. mesenteroides*, however, utilizes both NAD^+ and $NADP^+$ equally well[12]. At high concentrations, NAD^+ is as effective as $NADP^+$ for the enzyme from mammary gland[4].

G6PDH from *L. mesenteroides* was isolated in crystalline form which was homogeneous by disc gel electrophoresis and results of sedimentation velocity analysis were reported[8,9]. Isaque *et al.*[13] reported the amino acid composition of the enzyme. Kinetic studies showed that NAD^+ and $NADP^+$ interact with different enzyme form[14]. Grove *et al.*[11] reported that the dissociation constant for $NADP^+$ is 3 μM and that for NAD^+ is 2.5 mM by the fluorescence quenching method. Milhausen *et al.*[10] showed that there is an essential lysine at the active site of the enzyme and that the function of this lysine is to bind G6P.

One of the authors (M. H. H.) screened a strain of *L. mesenteroides* producing higher activity of G6PDH from a Korean Kimchi[15]. From this strain we have purified previously G6PDH by using a Cibacron Blue F3GA-Sepharose 4B affinity chromatography and showed the homogeniety of the enzyme preparation by a 5.0 % polyacrylamide gel electrophoresis[16].

In the present paper we report the result of characterization of G6PDH from *L. mesenteroides* No. 20-2-10.

MATERIALS AND METHODS

Glucose 6-phosphate Dehydrogenase Purification. G6PDH purified by an affintity chromatography[16] from *L. mesenteroides* No. 20-2-10 was used throughout this work.

Enzyme Assay. Enzyme assays were performed in a Beckman Spectrophotometer Model 25 which was kept at 30 °C with a thermocirculatory cell compartment by the method of Olive *et al.*[8]

Standard assay mixture contained the following components in a final volume of 3.0 m*l*: 33 m*M tris*-HCl buffer, pH 7.8: 3.3 m*M* G6P and either 2.5m*M* NAD^+ or 0.16m*M* $NADP^+$ (neutralized to pH 7.0). The reaction was followed

G6PDH : Glucose 6-phosphate dehydrogenase
G6P: Glucose 6-phosphate
NAD^+ and NADH: Oxidized and reduced nicotinamide-adenine dinucoleotide
$NADP^+$ and NADPH: Oxidized and reduced nicotinmide-adenine dinucleotide phosphate
K_{G6P}: Michaelis constant for glucose 6-phosphate of either NAD^+- or NAD^+-linked reaetion
K_{NAD}: Michaelis constant for NAD^+
K_{NADP}: Michaelis constant for $NADP^+$
α: The ratio of αK_{NAD} to K_{NAD} or αK_{NADP} to K_{NADP}
V_m: Maximum velocity as substrates are varied.

by measuring the increase in absorbancy at 340 nm with time. When $NADP^+$ was used instead of NAD^+, the ratio of activity with NAD^+ to $NADP^+$ was constant at 1.8. One unit of enzyme activivity was defined as the amount of enzyme required to catalyze the reduction of 1 μmole of NAD^+ per min at 30 °C if not specified.

The steady state kinetic analysis were carried out by the enzyme activity under the standard assay condidtion. For the series of experiments conducted at various pH values, the approximately diluted enzyme was assayed on the day of the experiment under the standard assay condition. The values for V_m obtained in all experiments were compared in the pH-V_m profile[18] after normalization. The concentration of the enzyme used did not exceed 10^{-4} times the concentration of substrates, fulfilling the steady state kinetic requirements. Since it was reported[14] that V_m was largely affected by changes in ionic strength, the calculated ionic strength of each buffer was fixed at the standard assay condition over the pH range employed; 16 m*M* succinic-NaOH buffer, pH 5.0: 18 m*M* phosphate buffer, pH 6.0; 22 m*M* *tris*-HCl buffer, pH 7.1: 33 m*M* *tris*-HCl buffer, pH 7.8: 150 m*M* *tris*-HCl buffer, pH 8.9: 32 m*M* glycin-NaOH buffer, pH 10.0.

It was necessary to determine approximate K_m values at varying pH prior to initial velocity experiments. This allowed experiments to be conducted by varying substrate concentrations over a range relative to each K_m, for example, 0.5 to 5 K_m.

Temperature effect on enzyme activity was determined under the standard assay condition at varying temperature from 10 to 60 °C.

Molecular Weight Determination. The apparent molecular weight of the enzyme was determined using Sephadex G-200 column according to the method of Andrews[17]. The column was calibrated with Blue Dextran 2,000 (M.W 2,000,000), glucose oxidase (M.W. 150,000), bovine serum albumin (M.W 67,000), peroxidase (M.W 40,000), and cytochrome C (M.W 13,000) as standard.

Modification of the Enzyme. In order to carry out photooxidation of the enzyme, the solution containing enzyme of 1 mg protein/m*l* and 0.01 % methylene blue in 10 m*M* phosphate buffer (pH 6.8) was irradiated with Kodak Carousel 600 Slide projector at the distance of 15 cm. The reaction mixture was stirred vigarously at the room temperature to saturate with atmospheric oxygen prior to the photooxidation process. The sample was kept in the dark before and after the irradiation. During the reaction 1 m*l* aliquots were taken at given times and remaining enzyme activity was assayed under the standard assay condition with appropriate dilution. A control containing the enzyme (1 mg protein/m*l*) and dye kept in the dark was analyzed simultaneously[19].

To alkylate the imidazole group of histidine, the reaction was performed by the procedure of Banazak[20]. The final reaction mixute contained 0.2 *M* bromoacetate and G6PDH (1mg protein/m*l*) in 1 *M* phosphate buffer (pH 6.9). During the course of the reaction, 1 m*l* aliquote were taken at given times and the remaining enzyme activity was determined under the standard assay conditions described previously.

Materials. NAD^+, $NADP^+$ and G6P were purchased from Sigma Chemical Co. (U.S.A.) and other chemicals were reagent grade.

RESULTS

Determination of Molecular Weight. *Fig.* 1 shows a plot of the elution volume from Sephadex G-200 column against logarithm of molecular weight of standard proteins according

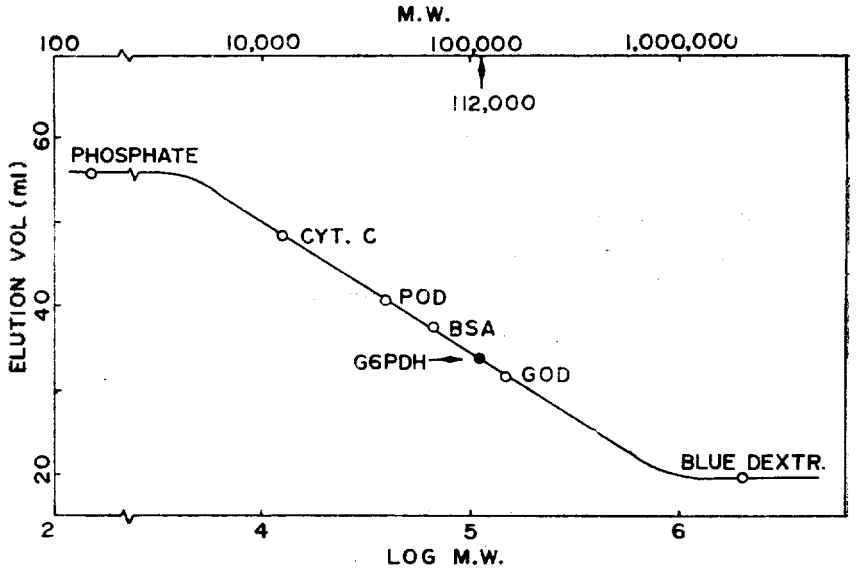


Fig. 1. Plots of elution volume, V_e against log (molecular weight) of standard materials on Sephadex G-200 column equilibrated with ammonium formate, pH 6.9. Molecular weight of glucose 6-phosphate dehydrogenase was intrapolated to be 112,000.

to the method of Andrew[17]. The molecular weight of G6PDH from *L. mesenteroides* was estimated to be 112,000 from the plot.

Initial Velocity The double reciprocal plots of initial velocity and substrate concentration for NAD^+-linked reation are illustrated in *Fig.* 2 & 3. The linear lines of the double reciprocal plots suggest that kintic reaction of GPDH from *L. mesenteroides* are consistent with a sequential mechanism and obeys the following equation:

$$v=V_m[NAD^+][G6P]/\{\alpha K_{NAD}K_{G6P}+K_{NAD}[G6P]+K_{G6P}[NAD^+]+[NAD^+][G6P]\}$$

The secondary plot of the apparent values of the slop and the intercept in the primary Lineweaver-Burk plots are also demonstrated to be linear as shown in *Fig.* 2 & 3.

The calculated kinetic constants for the NAD^+-linked reaction obtained from these secondary plots are as follows: K_{G6P} 53.7 μM, K_{NAD} 115.2 μM and αK_{NAD} 707.2 μM.

Initial velocity studies were also carried out for the $NADP^+$-linked reaction at the same coditions of NAD^+-linked reaction. The double reciprocal plots of initial velocity and substrate concentrartion was identical to those for the NAD^+-linked reaction as illustrated in *Fig.* 2 & 3, except the intersection point was on the abscissa for the $NADP^+$-linked reation. The ratio of αN_{NADP} to K_{NADP}, or α (see Appendix), is, therefore, unit where as that of αK_{NAD} to K_{NAD} is 6.14.

The kinetic constants for the reaction were estimated to be K_{G6P} 76.9 μM, K_{NADP} 7.46 μM, and αK_{NADP} 7.14 μM. The data obtained from the experiment are summarized in *Table* 1.

pH Effect. The pH dependent kinetic constants are summarized in *Table* 2. The pH profiles for the kinetic constants obtained from the series of experiments are shown in *Fig.* 4 (A~D). The results indicate that the G6PDH reaction is associated with at least 2 ionizing groups: pKb=9.0~9.5 and pKa=7.2.

Temperature Effect. The temperature-activity profile showed that an optimum temperature was 50 °C (*Fig.* 5). The Arrhenius plot

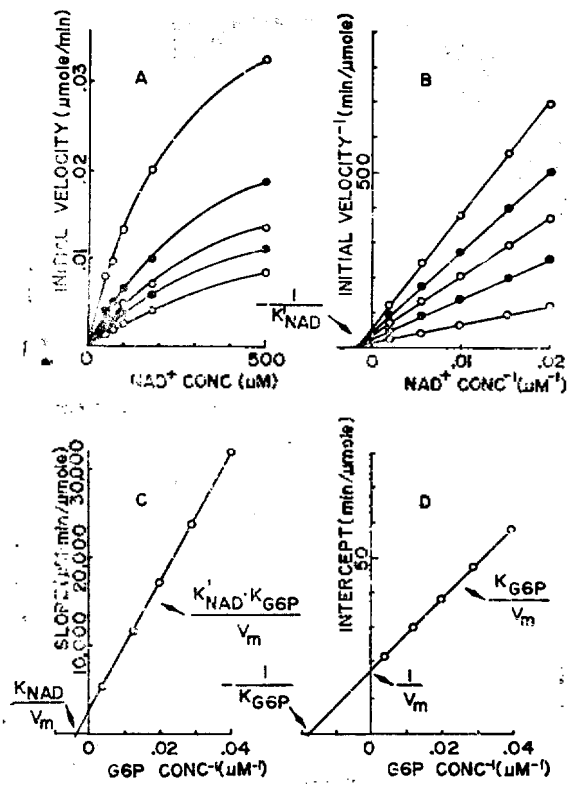


Fig. 2. Effect of substrate concentration on NAD-linked glucose 6-phosphate dehydrogenase reaction in 33 m*M tris*-HCl buffer, pH 7.8.

A. Initial velocity versus NAD^+ concentration at various constant glucose 6-phosphate concentration of 25, 35, 50, 80, 250 μM.

B. Primary double reciprocal plot of initial velocity versus NAD^+ concentration at various constant glucose 6-phosphate concentration.

C. Secondary plot of slopes of B against reciprocal of glucose 6-phosphate concentration

D. Secondary plot of intercepts of B against reciprocal of glucose 6-phosphate concentration

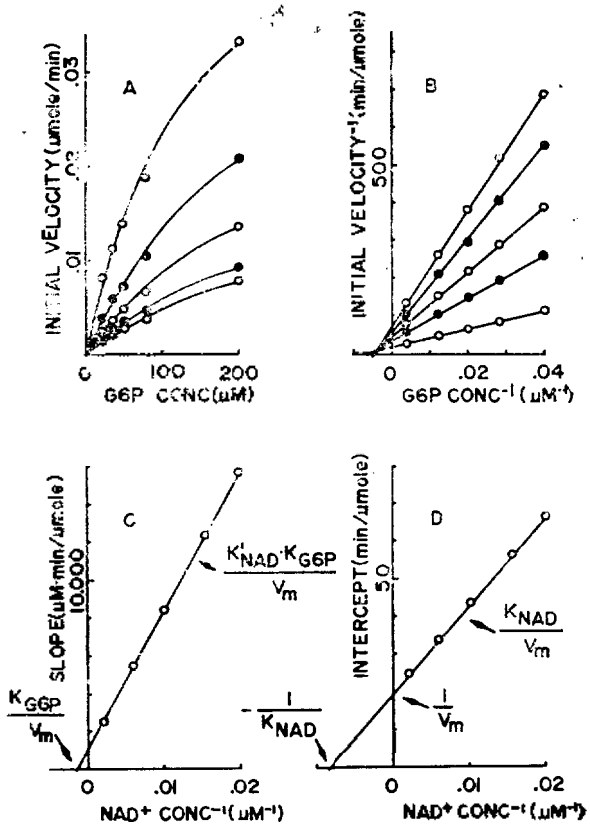


Fig. 3. Effect of substrate concentration on NAD^+-linked glucose 6-phosphate dehydrogense reaction in 33 m*M tris*-HCl buffer, pH 7.8.

A. Initial velocity versus glucose 6-phosphate concentration at various constant NAD^+ concentration of 50, 65, 100, 175, 500 μM.

B. Primary double reciprocal plot of initial velocity versus glucose 6-phossphate concentration at various constant NAD^+ concentration

C. Secondary plot of slopes of B against reciprocal of NAD^+ concentration

D. Secondary plot of intercepts of B against reciprocal of NAD^+ concentration

demonstrated a sharp curvature at 50°C demonstrating thermodenaturation[21] of the enzyme. The activation energy, E_a, of the G6PDH reaction was calculated to be 8.36 kcal/mole at a low temperature range and the heat of inactivation was −58.2 kcal/mole at a high temperature range.

Chemical Modification. Studies on carboxymethylation of the enzyme with bromoacacetic acid and photooxidation in the presence of a photosensitizing dye, methylene blue, were carried out to elucidate possible active amino acid residues of G6PDH from *L. mesenteroides*. Photooxidation inactivated G6PDH activity as

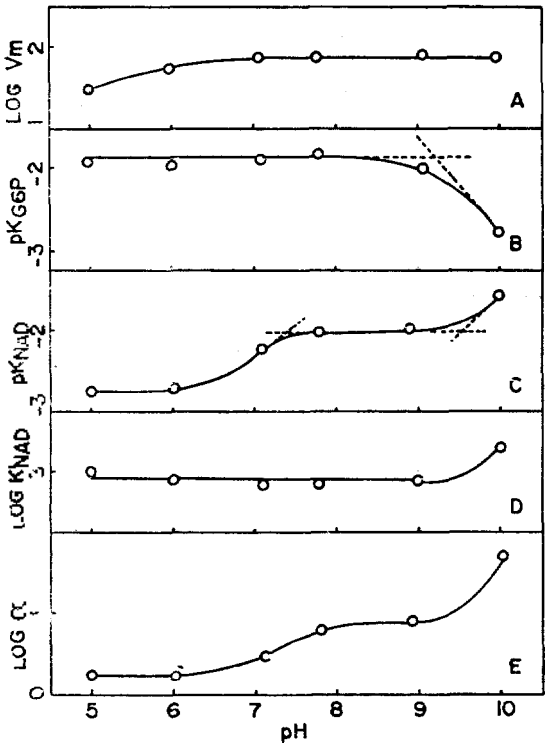


Fig. 4. The effect of pH on kinetic constants of the NAD$^+$-linked reaction. Buffers used are; 16 m*M* succinic-NaOH, pH 5.0; 18 m*M* phosphate, pH 6.0; 22 m*M* *tris*-HCl, pH 7.1; 33 m*M* *tris*-HCl, pH 7.8; 150 m*M* *tris*-HCl, pH 8.9; 32 m*M* glycin-NaOH, pH 10.0. A. Plot of log V_m *vs.* pH; B. plot of pK_{G6P} *vs.* pH; C. plot of pK_{NAD} *vs.* pH; D. plot of log K'_{NAD} *vs.* pH; E. plot of log α *vs.* pH.

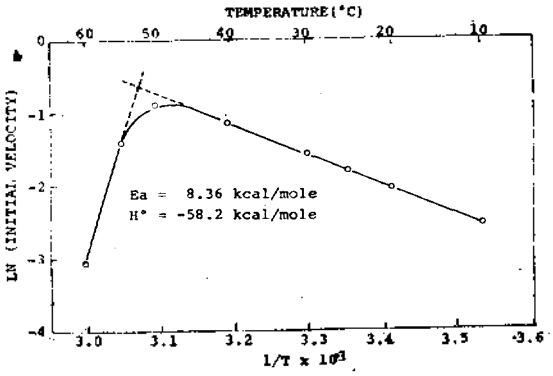


Fig. 5. Temperature dependency of glucose 6-phosphate dehydrogenase reaction: Arrehnius plot.

Table 1. Kinetic constants of NADP$^+$- and NAD$^+$-linked reaction of glucose 6-phosphate dehydrogenase from *L. mesenteroides*.

Substrate	K_m (μM)	$K_{NAD(P)}$ (μM)	α
NADP$^+$	7.46	7.14	0.96
Glucose 6-phosphate[a]	7.6	—	—
NAD$^+$	115.2	707.2	6.14
Glucose 6-phosphate[b]	53.7	—	—

All values were determined in 33 m*M* *tris*-HCl, pH 7.8
[a] Values determined in the NADP$^+$-linked reaction
[b] Values determined in the NAD$^+$-linked reaction

shown in *Fig.* 6. The inactivation kinetics was the first order with the rate constant, -0.775 min^{-1}. The half-life of the inactivation was 53.7 sec.

The first order rate constant of the inactivation by carboxymethylation was determined to be -0.037 hr^{-1} and the half-life was 18.6 hr under the given condition (*Fig.* 7).

The results of photooxidation and carboxymethylation experiments suggest that imidazole group of histidine is involved in the G6PDH reaction.

DISCUSSION

The apparent molecular weight of G6PDH from *L. mesenteroides* was determined to be 112,000 using Sephadex G-200 Column. The molecular weight of G6PDH varies depend on the source of the enzyme; 190,000[22] or 105,000[23] from human erythrocyte, 128,000 from yeast[24], 63,300 from rat mammary gland[25], 103,700 from *L. mesenteroides*[3]. Isaque *et at.*[13] reported that G6PDH from *L. mesenteroides* is consisted with two subunits whose molecular weight is 55,000. Levy *et al.*[3] also reported that NADP$^+$ caused polymerization of the enzyme isolated from the mammary gland whose apparent molecular weight was 63,300. The moecular weight of G6PDH obtained in this study, is consistant

Table 2. Kinetic constants at various pH.

Buffer	V_m (μmole/min)	K_{G6P} (μM)	K_{NAD^+} (μM)	αK_{NAD^+} (μM)	α
pH 5.0, succinic-NaOH, 16 mM	0.031	81.60	567.0	991.9	1.75
pH 6.0, phosphate, 18 mM	0.054	96.84	494.9	824.9	1.67
pH 7.1, *tris*-HCl, 22 mM	0.082	66.70	224.4	673.4	3.0
pH 7.8, *tris*-HCl, 33 mM	0.076	53.65	115.2	707.2	6.14
pH 3.9, *tris*-HCl, 150 mM	0.080	100.98	102.1	816.6	8.00
pH 10.0, glycine-NaOH, 32 mM	0.074	606.10	40.4	2020.0	50.0

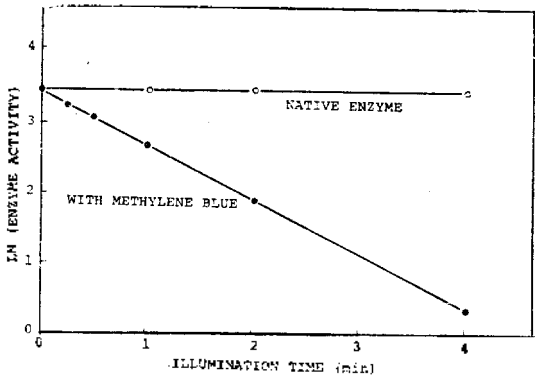


Fig. 6. Rate of photooxidation of glucose 6-phosphate dehydrogenase with 0.01 % methylene blue in 10 mM phosphate buffer, pH 6.8: replot according to the first order kinetics.

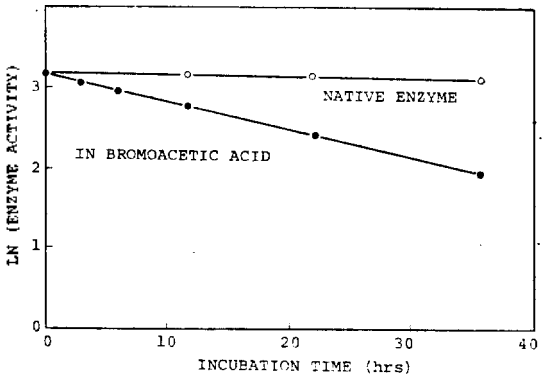


Fig. 7. Time course of carboxymethylation of glucose 6-phosphate dehydrogenase with 0.2 M bromoace tate in 1 M phosphate buffer, pH 6.9: replot according to the first order kinetics.

with value for a dimers. The molecular weight of monomer units can be estimated to be about 56,000, which is well agreeable with the report.

As shown in *Table* 1, the kinetic constants for the NAD^+-linked and the $NADP^+$-linked G6PD H reaction were in fairly good agreement with the values reported by others[8,14], except the early result by DeMoss *et al.*[7] This difference might be resulted from the enzyme preparation which was partially purified. Grove *et al.*[11] reported that the dissociation constant for NAD^+ determined from fluorescence quenching measurement was 2.5 mM, which was 3.5 times larger than the value of 0.707 mM obtained by this experiment. It was suggested that G6P which was present in the kinetic studies but not in the binding studies with fluorometer, acted as an allostetric activator and thus played an important role in regulating the coenzyme affinity iitry to the enzyme.

For the $NADP^+$-linked reaction. Olive *et al.*[14] proposed the ordered sequential mechanism with the oxidized coenzyme being bound first and the reduced coenzyme released last. They also elucidated the reaction mechanism for the NAD^+-linked reaction by the experiment of initial velocities, product inhibiton, alternate product inhibition, and alternate substrates, in which NAD^+ reacts with the modified form of enzyme (E_0) and, thereforore, the isomeriza-

tion of free enzyme (E_4) is essential. The steady state rate equation based upon these mechanism is derived (see Appendix) and the kinetic parametere are listed in *Table* 3.

However, they did not mention a possible mechanism of the involvement of the isomerization step for the $NADP^+$-linked reaction as shown in the NAD^+-linked reaction. There is no reason why the isomerization step should not be considered for the $NADP^+$-linked reaction. It is also theoretically possible to interpret the kinetic pattern of the $NADP^+$-linked reac tion with the isomerization step involved, if α constant is unity. In fact, for the $NADP^+$-linked reaction, α is unity, which means that dissociation rate constant (k_{-1}) is identical with molecular reaction velocity. On the other hand, α value is 6, which means that the dissociation rate constant is much larger than molecular reaction velocity, in the NAD^+-linked reation.

The meaning of α can be interpreted as the ratio of E_0-NAD dissociation rate constant (k_{-1}) to molecular reaction velocity. Therefore the α term can be simplified as being either k_{-1}/k_3, k_{-1}/k_4, or k_{-1}/k_5 depending on a possible rate-limiting step which is difficult to be determined experimentally.

From these interpretation, G6PDH reaction may be associated with an isomerization step for both $NADP^+$-linked reactions, which may have different values of rate constants, k_{-1}, k_3, k_4, or k_5. The position of an intercepting point on a primary double reciprocal plot can also be determined by the value of α. The point will be found on the abscissa only when α value is unity.

The elucidation of the effect of pH change on the kinetic constants of a reaction can provide useful guides for the elucidation of ionizing groups. V_m is unaltered between pH 6.5 and pH 10.0 although there is a small decrease

Table 3. Steady state kinetic parameters.

General expression	Steady state expression
V_m	$\frac{k_3k_4k_5E_0}{k_4k_5+k_3k_5+k_3k_4}$
K_{G6P}	$\frac{(k_3+k_{-2})\ k_4k_5}{k_2k_3k_4+k_2k_3k_5+k_2k_4k_5}$
K_{NAD}	$\frac{(k_5+k_{-5})\ k_3k_4}{k_1k_3k_4+k_1k_3k_5+k_1k_4k_5}$
K_{G6P}/V_m	$\frac{k_3+k_{-2}}{k_2k_3E_0}$
K_{NAD}/V_m	$\frac{k_5k_{-5}}{k_1k_5E_0}$
αK_{G6P} (K'_{G6P})	$\frac{k_{-1}(k_2+k_{-2})}{k_2k_3}$
αK_{NAD} (K'_{NAD})	$\frac{k_{-1}}{k_1}\left(1+\frac{k_{-5}}{k_5}\right)$
α	$k_{-1}\left(\frac{1}{k_3}+\frac{1}{k_4}+\frac{1}{k_5}\right)$
αV_m	$k_{-1}\ E_0$

below pH 6.5, which is insufficient to permit assigning of any pK values (*Fig.* 4A).

The logα-pH and the log αK_m-pH profiles (*Fig.* 4 D&E) are, therefore, due to the pH dependency of E_0, since the k_{-1} is pH independent.

This process is also probably linked with isomerization reaction of enzyme, the k_5 step.

The analysis of the pH-K_{NAD} profile (*Fig.* 4B) indicates that the two ionizing groups, *pKa* is 7.2 and *pKb* is 9.6 are associated with the interaction of substrate with enzyme. There is a clear evidence that a group on the enzyme with a *pKb* of approximately 9.0 is involved in the binding of G6P. The results suggest that the first group whose *pKa* is 7.2 may beean imidazole of histidine while the second group whose *pKb* is 9.0 ~9.6 is an ϵ-amino group of lysine as suggested by Milhausen *et al.*[10] and Olive *et al.*[14] They reported that the participating group on the *L. mesenteroides* G6PDH in binding of G6P was ϵ-NH_2 of lysine, whose pK was about 8.9. Furthermore, Isaque *et al.*[13] reported that G6PDH from *L. mesenteroides* was completely

devoid of cystein or cystine in its amino acid composition.

In this experiment, therefore, it was attempted to obtain further evidence for the identity of a group on the enzyme whose *pKa* is near 7.2, which could be involved in the interaction of NAD^+ to enzyme. First, methylene blue-sensitized photooxidation was employed. Of the 19 naturally occurring amino acids only five (tryptophan, tyrosine, methionine, cysteine and histidine) are readily susceptible to photodynamic degradation at pH value of 4~10. Its specificity for the various aromatic amino acid side chain is partially determined by pH. Oxidation of histidine usually takes place most rapidly at neutral pH. Trptophan and methionine are readily oxidized below pH 4.0. Tyrosine is most reactive at higher pH, where it is anionized. It has been reported that the quantum yield of photooxidation of tyrosine sensitized by methylene blue at pH 7.0 was zero whereas histidine was remarkable[25]. Experimental data was well fitted to the unimolecular first order kinetics.

Haloacetate are the most widely used protein alkylating agents. The incorporation of the carboxymethyl group into various side chains, such as those of cysteine, histidine, methionine and lysine is readly affected by various conditions. Since the added carboxymethyl group is quite similar to structures already present in proteins, being itself identical to the side chain of aspartic acids, its addition to the native protein often has little effect on the stability of the protein. Therefore, the interpretation of the loss of biological activity after carboxymethylation, for example, in terms of modifications of an active center is relatively secure.

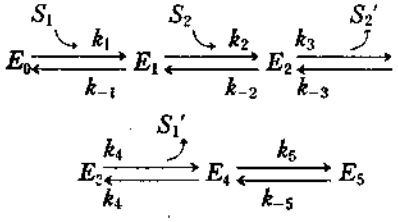
Of the several nucleophilic groups commonly found in proteins which are known to react with haloacetates, sulfhydryl groups are intrinsically the most reactive. The reactivity increases with pH since the anion is the reactive group. But it has been reported that G6PDH from *L. mesenteroides* have no cysteine residues[13].

Therefore, the possible reactivity of sulfhydryl group with haloacetate is excluded. At pH 5.5 and slightly above, where imidazole group are at least partially unprotonated, their modification is sufficiently fast to be the principal reaction in those proteins not having sulfhydryl group.

From these results, kinetics, photooxidation, carboxymethylation, it may be concluded that the imidazole of histidine is concerned with the interaction of NAD^+ to enzyme.

APPENDIX

The following two conditions are assumed for the G6PDH reaction as shown in *Scheme* 1. (1) 5 step ordered reaction and (2) isomerization of free enzyme involved in NAD^+ and NADH binding.



Scheme 1.

where S_1=NAD^+, S_2=G6P, S_1'=NADH, and S_2'=6PGL. A five step reversible reaction can be written as Eq. (1),

$$\frac{E_0}{v}=\frac{1}{\bar{v}}=S_1(R_1+R_2+R_3+R_4+R_5)+S_2+S_3+S_4+S_5=S_1\left(\sum_{i=1}^{5}S_i\right)+\sum_{j=2}^{5}R_j \qquad (1)$$

where

$$S_1=\frac{1}{k_1[S_1]}+\frac{k_{-1}}{k_1k_2[S_1][S_2]}+\frac{k_{-1}k_{-2}}{k_1k_2k_3[S_1][S_2]}$$

$$+\frac{k_{-1}k_{-2}k_{-3}[S_2']}{k_1k_2k_3k_4[S_1][S_2]}$$
$$+\frac{k_{-1}k_{-2}k_{-3}k_{-4}[S_1'][S_2']}{k_1k_2k_3k_4k_5[S_1][S_2]} \quad (2)$$

$$S_2=\frac{1}{k_2[S_2]}+\frac{k_{-2}}{k_2k_3[S_2]}+\frac{k_{-2}k_{-3}[S_2']}{k_2k_3k_4[S_2]}$$
$$+\frac{k_{-2}k_{-3}k_{-4}[S_2'][S_1']}{k_2k_3k_4k_5[S_2]} \quad (3)$$

$$S_3=\frac{1}{k_3}+\frac{k_{-3}[S_2']}{k_3k_4}+\frac{k_{-3}k_{-4}[S_2'][S_1']}{k_3k_4k_5} \quad (4)$$

$$S_4=\frac{1}{k_4}+\frac{k_{-4}[S_1']}{k_4k_5} \quad (5)$$

$$S_5=\frac{1}{k_5} \quad (6)$$

and

$$\sum_{i=1}^{5}R_i=\{k_1k_{-2}k_{-3}k_{-4}k_{-5}[S_1][S_1'][S_2']$$
$$+k_1k_2k_{-3}k_{-4}k_{-5}[S_1][S_2][S_1'][S_2']$$
$$+k_1k_2k_3k_{-4}k_{-5}[S_1][S_2][S_1']+k_1k_2k_3k_4k_{-5}$$
$$\cdot[S_1][S_2]+k_1k_2k_3k_4k_5[S_1][S_2]\}$$
$$/\{k_1k_2k_3k_4k_5[S_1][S_2]-k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}$$
$$\cdot[S_1'][S_2']\} \quad (7)$$

For the initial velocity study, $[S_2']$ and $[S_1']$ terms are negligible since there is no $[S_2']$ and $[S_1']$ formed at $t=0$.

Hence, Eq. (2)$\sim$(7) can be simplified as Eq. (8)$\sim$(13), respectively.

$$S_1=\frac{1}{k_1[S_1]}\left\{1+\frac{k_{-1}}{k_2[S_2]}+\frac{k_{-1}k_{-2}}{k_2k_3[S_2]}\right\} \quad (8)$$

$$S_2=\frac{1}{k_2[S_2]}\left\{1+\frac{k_{-2}}{k_3}\right\} \quad (9)$$

$$S_3=\frac{1}{k_3} \quad (10)$$

$$S_4=\frac{1}{k_4} \quad (11)$$

$$S_5=\frac{1}{k_5} \quad (12)$$

and

$$\sum_{i=1}^{5}R_i=\{k_1k_2k_3k_4k_{-5}[S_1][S_2]+k_1k_2k_3k_4k_5$$
$$\cdot[S_1][S_2]\}/k_1k_2k_3k_4k_5[S_1][S_2]$$
$$=\frac{k_{-5}}{k_5}+1 \quad (13)$$

By substituting Eq. (8)$\sim$(13) in Eq. (1), one obtains Eq. (14).

$$\frac{E_0}{v}=\frac{1}{\bar{v}}=\frac{1}{[S_1]}\left\{\frac{1}{k_1}+\frac{k_{-1}}{k_1k_2[S_2]}\right.$$
$$+\frac{k_{-1}k_{-2}}{k_1k_2k_3[S_2]}+\frac{k_{-5}}{k_1k_5}+\frac{k_{-1}k_{-5}}{k_1k_2k_5[S_2]}$$
$$\left.+\frac{k_{-1}k_{-2}k_{-5}}{k_1k_2k_3k_5[S_2]}\right\}+\left\{\frac{1}{[S_2]}\left\{\frac{1}{k_2}+\frac{k_{-2}}{k_2k_3}\right\}\right.$$
$$\left.+\left\{\frac{1}{k_3}+\frac{1}{k_4}+\frac{1}{k_5}\right\}\right. \quad (14)$$

Thus,

$$\frac{1}{v}=\frac{1}{E_0[S_1]}\left\{\left\{\frac{k_{-1}}{k_1k_2}+\frac{k_{-1}k_{-2}}{k_1k_2k_3}+\frac{k_{-1}k_{-5}}{k_1k_2k_5}\right.\right.$$
$$\left.+\frac{k_{-1}k_{-2}k_{-5}}{k_1k_2k_3k_5}\right\}\frac{1}{[S_2]}+\frac{1}{k_1}+\frac{k_{-5}}{k_1k_5}\Big\}$$
$$+\frac{1}{E_0[S_2]}\left\{\frac{1}{k_2}+\frac{k_{-2}}{k_2k_3}\right\}$$
$$+\frac{1}{E_0}\left\{\frac{1}{k_3}+\frac{1}{k_4}+\frac{1}{k_5}\right\} \quad (15)$$

From a double reciprocal plot of Eq. (15), one obtains the following expressions for slope and intercept;

$$\text{Slope}=\frac{1}{E_0[S_1]}\left\{\frac{k_{-1}}{k_1k_2}+\frac{k_{-1}k_{-2}}{k_1k_2k_3}\right\}\left\{1+\frac{k_{-5}}{k_5}\right\}$$
$$+\frac{1}{k_1E_0}\left\{1+\frac{k_{-5}}{k_5}\right\} \quad (16)$$

$$\text{Intercept}=\frac{1}{E_0[S_2]}\left\{\frac{1}{k_2}+\frac{k_{-2}}{k_2k_3}\right\}$$
$$+\frac{1}{E_0}\left\{\frac{1}{k_3}+\frac{1}{k_4}+\frac{1}{k_5}\right\} \quad (17)$$

From a secondary plot of slope *vs.* $1/[S_1]$ and intercept *vs.* $1/[S_2]$ one can define K_{NAD}, K_{G6P}, K'_{NAD}, and V_m, since the initial velocity can also be expressed as follow:

$$\frac{1}{v}=\frac{1}{[NAD^+]}\left\{\frac{\alpha K_{G6P}\cdot K_{NAD}}{[G6P]\cdot V_m}+\frac{K_{NAD}}{V_m}\right\}$$
$$+\left\{\frac{K_{G6P}}{[G6P]\cdot V_m}+\frac{1}{V_m}\right\} \quad (18)$$

where the values of slope and intercept can be expressed as Eq. (19) and (20), respectively.

$$\text{Slope} = \frac{1}{[\text{G6P}]} \cdot \frac{\alpha K_{\text{NAD}} \cdot K_{\text{G6P}}}{V_m} + \frac{K_{\text{NAD}}}{V_m} \tag{19}$$

$$\text{Intercept} = \frac{1}{[\text{G6P}]} \cdot \frac{K_{\text{G6P}}}{V_m} + \frac{1}{V_m} \tag{20}$$

By comparing Eq. (16) and (17) with Eq. (19) and (20), respectively, various steady-state expressions for the kinetic parameters can be derived as seen in *Table* 3.

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