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The Specificity of Induced Conformational Changes. The Case of Yeast Glyceraldehyde-3-phosphate Dehydrogenase†

L. D. Byers and D. E. Koshland, Jr.*

ABSTRACT: The specificity of induced conformational changes and of the probes used to detect them has been investigated in yeast glyceraldehyde-3-phosphate dehydrogenase. Cyanylation of the active-site SH groups in two of the four identical subunits of glyceraldehyde-3-phosphate dehydrogenase has no effect on reactivity of the unmodified SH groups toward the cyanylating reagent (2-nitro-5-thiocyanobenzoic acid, NTCB) but results in total loss of catalytic activity. Cyanylation of the dicarboxamidomethylated enzyme was four orders of magnitude slower than with the unmodified enzyme in contrast to cyanylation of the dicyanylated enzyme. Cyanylation by NTCB as well as alkylation by iodoacetate and acylation with β-(2-furyl)acryloyl phosphate are enhanced in the presence of NAD^+ while alkylation by iodoacetamide is inhibited by NAD^+. In the absence of NAD^+, hydrolysis of the acylated enzyme is faster than phosphorolysis while the reverse is true in the presence of NAD^+. NAD^+ accelerates hydrolysis of the 3-phosphoglyceroylated enzyme about 60-fold but decreases the rate of hydrolysis of the furylacryloylated enzyme by a factor of 17. Other examples of the specificity of the induced conformational changes and the probes are described. The conformational changes induced by NAD^+ make the protein specifically reactive toward its physiological substrates and less reactive toward extraneous competing compounds.

The role of conformational changes induced by substrates, inhibitors, and activators is now well established. These changes in protein structure play a role in enzyme specificity and enzyme regulation and in the cooperative interaction between subunits (Koshland and Neet, 1968). Nevertheless certain features of these conformational changes continue to raise questions. For example, a two-state model (Monod et al., 1965) suggests that a particular reactive group is either in one structural position or another and hence will exist in two different states of reactivity. On the other hand, a ligand induced model (Koshland et al., 1966) suggests that the position of the reactive groups will depend on the ligand inducing the conformational change and hence reactivity may depend specifically on the ligand which is binding to the protein. Secondly, different probes may give different signals when changes in structural alignments occur. For example, a probe which responds to hydrophobicity attached to a group which moves from one hydrophobic environment to another would signal "no net change in conformation", whereas one which measures accessibility to a chemical reagent would signal a significant change.

To throw further light on these questions, yeast glyceraldehyde-3-phosphate dehydrogenase has been examined. Extensive studies on the cooperativity properties of the enzyme (Kirschner et al., 1966, 1971; Chance and Park, 1967; Cook and Koshland, 1970; Kirschner, 1971; Sloan and Velick, 1973; Stallcup and Koshland, 1973a,c) and the mechanism of action of GPD from a variety of species (Krimsky and Racker, 1963; Trentham, 1971; Orsi and Cleland, 1972; Harrigan and Trentham, 1973, 1974) have been published.

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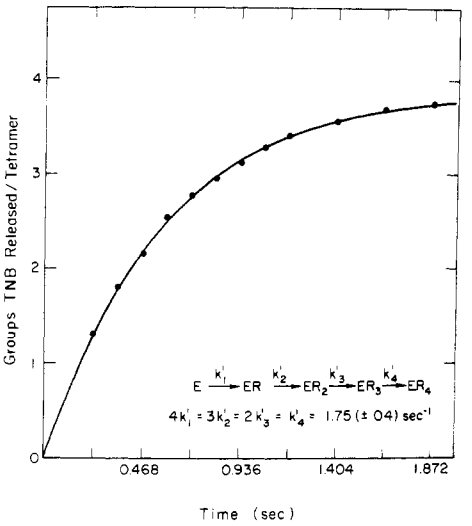


FIGURE 1: NTCB reaction with GPD. Data obtained from oscilloscope trace from stopped-flow spectrophotometer. E = 1.49 μM, NTCB = 1.92×10^{-4} M, 0.025 M bicine, 0.025 M NaCl, and 0.25 mM EDTA (pH 8.5), 25 ° (final conditions after mixing). Solid line is theoretical curve for a single exponential process (i.e., $[TNB]_t = 3.9(1 - e^{-1.749t})$.

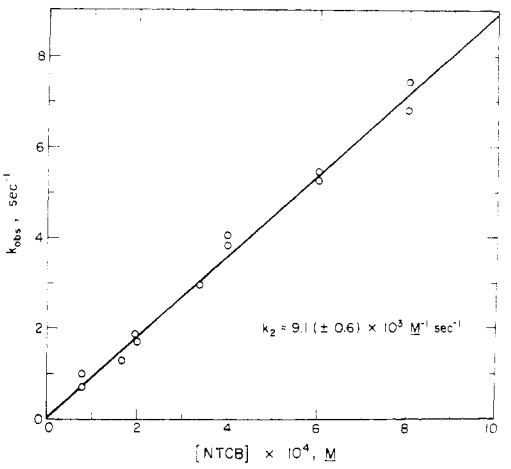


FIGURE 2: NTCB reaction with GPD as function of NTCB concentration. 1.5 mM enzyme, 0.025 M bicine, 0.025 M NaCl, and 0.25 mM EDA (pH 8.5), 25°. Solid line is a least-squares fit of the data yielding a second-order rate constant of 9.1 (±0.5) × 10^3 M^{-1} sec^{-1}.

The amino acid sequence of the yeast enzyme is known (Jones and Harris, 1972) and the three-dimensional structure for the lobster muscle enzyme (Buehner et al., 1973, 1974) has been delineated. Moreover the fact that negative cooperativity in the yeast enzyme may operate on the catalytic mechanism via the active site sulfhydryl residue makes the enzyme an object of particular interest (Stallcup and Koshland, 1973a–c; Levitzki and Koshland, 1975). Therefore, in this study the SH group of the active site has been examined in a variety of tests involving (a) the reactivity of the cysteine-149 group toward alkylating and acylating reagents, (b) the reactivity of the cysteine-149 group as a leaving group in deacylation, and (c) the influence of NAD^+ on each of these reactions.

Materials and Methods

Yeast GPD[1] was prepared as described previously (Stallcup et al., 1972). The enzyme was free of NAD^+ as estimated by an A_{280}/A_{260} ratio of 2.08–2.12. The samples employed for these studies had a specific activity of 100–110 μmol of NADH produced per min per mg of protein (Stallcup et al., 1972). Yeast 3-phosphoglycerate kinase was obtained from Boehringer Mannheim and desalted before use (specific activity for ATP production ~4000 units/mg).

G3P was a racemic mixture prepared from the barium salt of the diethyl acetal obtained from Sigma Chemical Co. All G3P concentrations referred to in this paper are for the D enantiomer which is assumed to be the only reactive component of the racemic mixture. Concentrations of G3P,[2] NAD^+, and AP-NAD^+ were determined by enzymic assay. The G3P solutions contained <0.01% free phosphate as estimated by the method of Hwang and Cha (1973). The nucleotides, NAD^+, NADH, ADP-ribose, and AP-NAD^+ were also obtained from Sigma. AP-NADH formation was followed at λ 365 nm with ϵ_{365} 7.8×10^3 M^{-1} cm^{-1} (Kaplan et al., 1956).

FAP was prepared and analyzed by the method of Malhotra and Bernhard (1968). NTCB was prepared by the method of Degani and Patchornik (1971). The potassium half-salt had mp 242–243° (lit. mp 243–245°). Anal. Calcd: C, 39.4; H, 1.44; N, 11.5; S, 11.3. Found: C, 39.5; H, 1.45; N, 11.5; S, 11.7. Concentrations were standardized by titration with cysteine. The reaction was followed by release of the thionitrobenzoate chromophore at 412 nm with ϵ_{412} 1.36×10^4 M^{-1} cm^{-1} (Ellman, 1959). Methyl phosphonate was prepared by the method of Crofts and Kosolopoff (1953). The product has mp 104–105° (lit. mp 104–105°). Buffers and reactants were prepared with Na^+ as the counterion. Unless otherwise indicated all reactions were carried out at 25° and pH 8.5 in bicine [*N,N*-bis(hydroxyethyl)glycine] buffer containing 0.25 or 0.43 mM EDTA. For the NTCB stopped-flow reactions the syringe containing enzyme was 0.05 M in bicine (0.5 mM EDTA) and the NTCB syringe contained 0.05 M NaCl (unbuffered) to avoid spectral effects of salt dilution during mixing. The final bicine concentration in these experiments was 0.025 M (pH 8.5). The tetracyanylated enzyme obtained under these conditions was stable with a half-life for regain of activity of about 20 hr.

The reactions with alternate nucleophiles were carried out in high bicine concentrations (0.425 M) to avoid ionic strength effects (under these conditions the reaction was

[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12]; G3P, glyceraldehyde 3-phosphate; ADP-ribose, adenosine 5′-diphosphoribose; AP-NAD^+, 3-acetylpyridine adenine dinucleotide (acetylpyridine analog of NAD^+); AD-NADH, reduced form of AP-NAD^+; FAP, β-(2-furyl)acryloyl phosphate; NTCB, 2-nitro-5-thiocyanobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; bicine, *N,N′*-bis(2-hydroxyethyl)glycine.

[2] It has been reported (Duggleby and Dennis, 1974) that G3P is unstable in 8.2–8.9 with a half-life of ~4 min at pH 8.2 (0.2 M Tris-HCl–2.44 mM D,L-G3P, 25°). We find no such instability of G3P when preincubated in assay mixtures (without enzyme) containing pyrophosphate (0.05 M), Tris-HCl (0.02 M), or bicine (0.05–0.5 M) buffers at pH 8.5. Indeed the half-life for aldehyde loss resulting from incubation of G3P (10^{-4} M) in bicine buffer (0.4 M, pH 8.5) is >30 hr at 25°. Therefore the aldehyde loss under the conditions of Duggleby and Dennis (1974) does not reflect the inherent instability of G3P but probably results from the high amine concentration (0.2 M Tris) and/or the high aldehyde concentration employed.

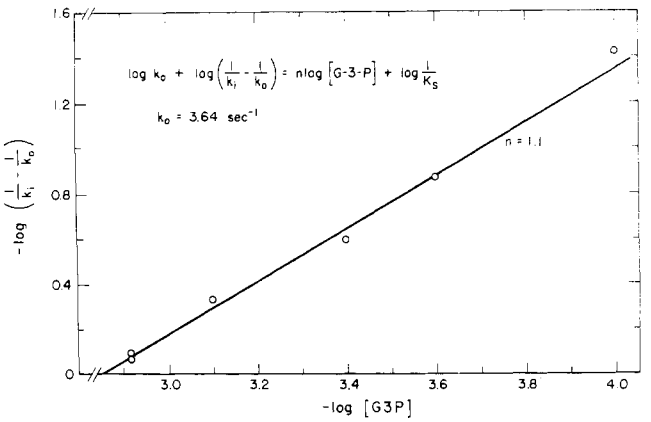


FIGURE 3: Hill plot for G3P protection of GPD against NTCB reaction. Reaction conditions are as in Figure 2, [NTCB] = 4×10^{-4} *M*. Solid line is for theoretical curve: $\log k_0 + \log [(1/k_i) - (1/k_0)] = n \log [G3P] + \log (1/K_s)$ (reaction first-order in NTCB, $k_i = 0$ at [G3P] = ∞; see Loftfield and Eigner, 1969; Koshland, 1970) where k_0 is the rate constant for cyanylation of the enzyme by NTCB in the absence of G3P ($k_0 = 3.64$ sec^{-1}), k_i is the rate constant for reaction in the presence of G3P, K_s is the dissociation constant of G3P from the enzyme ($K_s = 9.5 \times 10^{-5}$ *M*), and n is the Hill coefficient ($n = 1.1$).

about two times faster than in the presence of 0.1 *M* bicine). The enzymic reaction was initiated by addition of G3P to an incubation mixture containing enzyme, NAD^+, and nucleophile. The reaction velocity was independent of the preincubation time (from 0.5 to 10 min).

Absorbancies were measured on a Zeiss PMQII spectrophotometer. Reactions were followed either on a thermostated Gilford 2000 recording spectrophotometer or on a Durrum Gibson stopped-flow spectrophotometer (optical path length = 2 cm) and recorded on a Tektronix 549 storage oscilloscope. First-order kinetic data were analyzed by a computer program for a least-squares reduction.

Results

Effects of NTCB. 2-Nitro-5-thiocyanobenzoic acid (NTCB) is an effective cyanylating agent for SH groups in proteins (Degani and Patchornik, 1974). The reactivity of apo-GPD with NTCB is shown in Figure 1. The enzyme reacts in a smooth pseudo-first-order process for at least 5 half-lives and 3.7 (±0.2) cyano groups are incorporated per mole of enzyme. The first-order rate indicates complete independence of reacting sites, i.e., the cyanylation of 1 SH group has no effect on the reactivity toward NTCB of the SH groups at any of the unmodified sites. This is in contrast to the patterns of most of the SH reagents which react with native yeast GPD. Iodoacetamide, iodoacetic acid, mercurials, and many acylating agents all show half-of-the-sites reactivity in which modification of the two subunits drastically reduced the reactivity of the remaining two subunits toward modifier (Stallcup and Koshland, 1973a). It is consistent, however, with the reaction of DTNB (von Ellenrieder et al., 1972; Stallcup and Koshland, 1973a) and *p*-nitrophenyl acetate (Stallcup and Koshland, 1973a) which have also been shown to react independently with all four subunits of the enzyme.

To determine the role of binding in the NTCB reaction, the concentration of NTCB was varied over a tenfold range and the pseudo-first-order rate constants were calculated. There is no indication of detectable saturation of the enzyme, indicating a K_d of greater than 2 m*M* (Figure 2). The second-order rate constant for the reaction of NTCB with the yeast enzyme is 9.1 (±0.06) $\times 10^3$ M^{-1} sec^{-1} at 25° and pH 8.5.

It might be argued that NTCB is acting with a different SH group than other alkylating agents, but this can be clearly eliminated by protection experiments with the substrate glyceraldehyde 3-phosphate (Figure 3) and by similar experiments with the acylated enzyme. G3P inhibits the rate, but not the extent, of the cyanylation reaction. This probably reflects formation of a thiohemiacetal with the active-site SH group. Segal and Boyer (1953) observed similar protection by G3P for the alkylation of the active-site SH group of yeast GPD by iodoacetate. Extrapolation of the observed cyanylation rate constant to [G3P] = ∞ indicates complete protection of GPD against cyanylation at saturating concentrations of the aldehyde. From this observation and the kinetics of the NTCB reaction (Figures 2 and 3) a dissociation constant of G3P from the enzyme, K_s = 9.5 (±0.5) $\times 10^{-5}$ *M*, is obtained.[3] [From ultracentrifugation studies Velick and Hayes (1953) obtained a dissociation constant of G3P from rabbit muscle GPD of ~4 $\times 10^{-5}$ *M* (pH 8.5, 4°).]

Apparently NTCB is reacting with the cysteine-149 at the active site, but either (a) the cyano group is not inducing the same conformation changes as do the other alkylating agents or (b) the NTCB reaction is insensitive to these changes. To decide between these alternatives, enzyme was treated with iodoacetamide until two groups per tetramer had been alkylated and was then treated with NTCB (2 m*M*). NTCB reacted with the two remaining unmodified SH groups about four orders of magnitude slower than with the native enzyme. Thus NTCB was able to "see" the conformational change induced by iodoacetamide. The lack of cooperativity in the reaction with the cyano group must, therefore, be caused by the failure of the cyano group to induce the same kind of conformational change as that induced by the other alkyl groups, mercurials, or acyl groups.

The stoichiometry of inactivation of the enzyme is shown in Figure 4. When an average of two groups are modified per tetramer the enzyme has only 16% of its native activity. This does not mean that the species ER_2 has 16% of activity of the native enzyme, but rather that the mixture in which an average of two groups per total protein are modified has 16% activity. Since the solution contains the molecular species E, ER, ER_2, ER_3, and ER_4 and each site acts independently it is possible to calculate the molecular species present at any point on the modification curve assuming the usual binomial probability distribution. The best fit to the experimental data is obtained by assuming that ER has 40% of the activity of the native protein and that ER_2, ER_3, and ER_4 are inactive. Thus the conformational change induced by the cyano group at one of the SH groups of the tetramer, which has no effect on the reactivity of neighboring chains with NTCB, has a profound effect on the enzymic activity. If there were no interactions between subunits, the ER species would be expected to have 75% of the activity of the native protein. If the induced conformational change merely turned off a neighboring subunit, the ER

[3] A lower limit for the second-order rate constant for G3P binding to the enzyme can be obtained by adding G3P and NTCB stimultaneously to GPD at $t = 0$. Even at the lowest G3P concentration investigated (2×10^{-4} *M*) the production of TNB followed a first-order single exponential process with a rate constant identical with that when G3P was preincubated with the enzyme for 30 min. This implies that G3P reacts with the enzyme faster than does NTCB and, thus, the "on" rate of G3P is greater than 5×10^4 M^{-1} sec^{-1}.

Table I: The Effect of NAD^+ on Some Reactions with the Cysteine-149 Residue of Yeast GPD.[a]

Reaction	NAD^+ Concn (m*M*)	$k_{(+NAD^+)}/k_{(-NAD^+)}$	n_H
E-$PG_{1.5}$ Hydrolysis[b]	1	60	
Phosphorolysis (10 m*M* PO_4^{2-})	1	$>4 \times 10^{6}$*	
Arsenolysis (25 m*M* AsO_4^{2-})	1	$>2.4 \times 10^{5}$*	
E-$FA_{1.8}$ Hydrolysis	1	0.06	
	∞	~0	1.9
Phosphorolysis (62 m*M* PO_4^{2-})	1.25	>0.44*	
Arsenolysis (25 m*M* AsO_4^{2-})	1.25	>0.53*	
FAP acylation			
Fast phase[c]	2	4.8[g]	
	∞	10.1	1.0
Slow phase[c]	2	4.0[g]	
	∞	4.0	1.0
NTCB cyanylation	∞	5.5	1.0
DTNB modification (20°)[d]	∞	0.6	
ICH_2CONH_2 alkylation	1	0.1	
$ICH_2CO_2^-$ alkylation[e]			
$\mu = 0$	1	6.3	
$\mu = 0.1$	1	15.8	
NADH bursts [E + NAD^+ G3P vs. E + G3P NAD^+][f]	∞	>20	

[a] NAD^+ effects on some reactions at the active site of yeast GPD. All reactions were carried out at pH 8.5 (0.4 *M* bicine–0.4 m*M* EDTA), 25°. The numbers indicated with an asterisk (*) are for the rate constants for phosphorolysis (or arsenolysis) in the presence of NAD^+, divided by the rate constant for *hydrolysis* in the absence of NAD^+ [which is greater than the phosphorolysis (or arsenolysis) rates in the absence of NAD^+]. These ratios are, therefore, lower limits for the indicated reactions. [b] Stallcup and Koshland (1973c), 0.05 *M* Tris-HCl–0.01 *M* EDTA (pH 8.5). [c] The acylation is biphasic (even at 10 m*M* NAD^+) with each phase having approximately equal amplitudes. This is observed when following the reaction at 360 nm (production of native GPD thiofurylacryloyl group), at 336 nm for the guanidine-HCl denatured enzyme (thiofurylacryloyl group production) and back titration of the denatured enzyme with DTNB (free SH group determination). [d] von Ellenrieder et al., 1972. The reaction with DTNB is biphasic and this inhibition by NAD refers to the fast phase. [e] The ionic strength effect is due entirely to the inhibition of alkylation of the apoenzyme. The rate of alkylation of GPD in the presence of 1 m*M* NAD^+ is unaffected by salt concentrations up to 0.3 *M* NaCl. [f] The rapid transient production of about 4 mol of NADH/tetramer is faster when the enzyme is preincubated with NAD^+ (for about 5 min) and the reaction initiated by addition of G3P than when the order of addition is reversed. When the NAD^+ analog, acetylpyridine adenine dinucleotide (AP-AD) is substituted for NAD^+ the ratio of AP-NADH production is about 23 at saturating concentrations of AP-AD. [g] Ratio of acylation rate of GPD + NAD^+ (2 m*M*) to acylation rate of GPD + AP-AD (2 m*M* acetylpyrimidine adenine dinucleotide), $k_{(NAD^+)}/k_{(AP\text{-}AD)}$, = 47 for the fast phase and 72 for the slow phase.

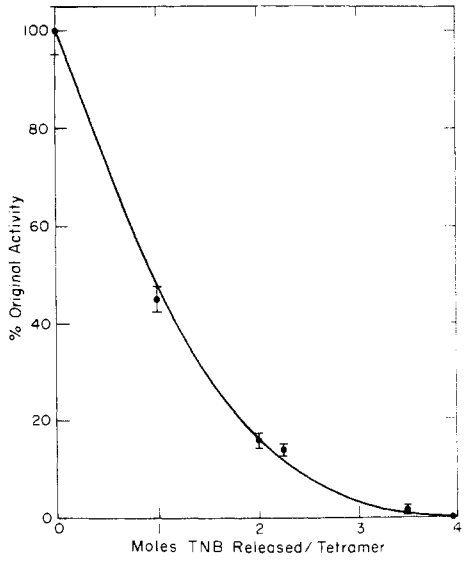


FIGURE 4: Stoichiometry of inactivation of GPD by NTCB. The solid curve is theoretical assuming the di-, tri- and tetracyanylated enzymes are inactive as dehydrogenases and the monocyanylated enzyme is 40% as active as the unmodified enzyme. The molecular distribution is calculated as a function of the moles of thionitrobenzoate (TNB) released per monomer (= K = stoichiometric amount of NTCB added) from the relation: $E(CN)_n/E_T = 4!K^n(1 - K)^{4-n}/n!(4 - n)!$ which is based on the assumption that the reactivity of each subunit is independent of the extent of modification of other subunits (see Figure 1).

species would have had 50% activity.

Role of NAD^+. Unlike G3P, which inhibits the reaction with NTCB, NAD^+ increases it. This contrasts to the slight inhibitory effect of NAD^+ on the reaction with DTNB (von Ellenrieder et al., 1972) and with iodoacetamide (Table I). The double reciprocal and Eadie–Hofstee plots indicate that at saturating amounts of NAD^+ the cyanylation reaction is 5.5 (±0.5) times faster in the presence of NAD^+ than in the absence of NAD^+ (cf. Figure 5). When the rate constant is studied as a function of NAD^+ concentration and the results are plotted on a Hill plot, no cooperativity in the NAD effect is detected, i.e., the Hill coefficient is 1.01. An NAD^+ binding constant of 1.9 (±0.2) $\times 10^{-4}$ *M* was found which agrees with the direct determination of binding constants to the apoenzyme (S. C. Mockrin, L. D. Byers, and D. E. Koshland, in preparation) and the kinetically determined K_m for the dehydrogenase and furoylacryloyl phosphate acylation reactions.

Studies of various analogs of NAD^+ show that they inhibit, rather than accelerate, the rate, but not the extent, of reaction with NTCB. Thus, ATP exhibits a K_i of 3.2 $\times 10^{-4}$ *M* and a Hill coefficient of 1.0. ADP-ribose ($K_i \geq 1.7 \times 10^{-3}$ *M*) and NADH ($K_i > 5 \times 10^{-3}$ *M*) also inhibit the cyanylation of GPD.

Role of NAD^+ in Deacylation. The NAD^+-increased rate of cyanylation or acylation could be due to its stabilizing the modified enzyme, i.e. the acyl- or cyanyl-enzyme. If so, it should also decrease the rate of the deacylation reaction. Accordingly the deacylation was studied with a variety of nucleophiles and the results are shown in Table I. It is readily seen that NAD^+ has a very specific effect on the various reactions. It accelerates the rate of deacylation of the phosphoglyceroyl-enzyme for its reaction with water, phosphate, and arsenate. Hence NAD^+ does not simply

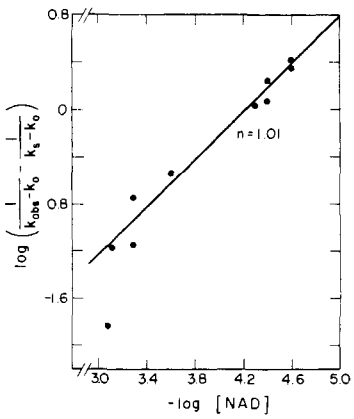


FIGURE 5: Hill plot for activation of the NTCB reaction by NAD^+. 1.5 μM enzyme, 1.75×10^{-4} M NTCB, 0.025 M bicine, 0.025 M NaCl, and 0.25 mM EDTA (pH 8.5), 25°. Solid line is for the theoretical curve: $\log (k_s - k_0) + \log [(1/(k_{obsd} - k_0)) - (1/(k_s - k_0))] = n \log [NAD^+] + \log K_s$, where k_0 is the rate constant for the reaction in the absence of NAD^+ ($k_0 = 1.60$ sec^{-1}), k_s is the rate constant for the reaction in the presence of saturating amounts of NAD^+ ($k_s = 8.0$ sec^{-1}), k_{obsd} is the observed rate constant, K_s is the dissociation constant of NAD^+ from the enzyme ($K_s = 1.8 \times 10^{-4}$ M), and n is the Hill coefficient ($n = 1.01$).

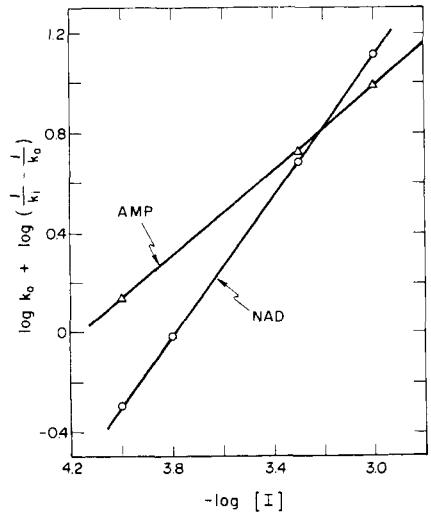


FIGURE 6: Hill plot for the protection of hydrolysis of GPD acylated with ~1.8 furylacryloyl groups/tetramer by NAD^+ (○) and by AMP (△). Reactions were carried out in 0.04 M bicine, 0.4 mM EDTA, and 0.1 M NaCl (pH 8.5), 25°. In the absence of NAD^+ the hydrolysis rate, k_0, is 0.078 min^{-1}. The rate constants for hydrolysis in the presence of inhibitor are k_i. The Hill coefficients are 1.9 for NAD^+ and about 0.87 for AMP.

thermodynamically stabilize the acyl intermediate. This is consistent with studies of GPD from several species where acylation of the active-site SH group *decreases* the binding of NAD^+ (Trentham, 1971; Malhotra and Bernard, 1973; Boers and Slater, 1973). On the other hand, the same NAD^+ decreases the rate of hydrolysis of the furylacryloyl-enzyme. Interestingly, a study of the cooperativity of NAD^+ in this process, illustrated in Figure 6, shows that the NAD^+ protection from hydrolysis has a Hill coefficient of 1.9 (while AMP, which also protects against hydrolysis, shows a Hill coefficient of about 1). This is in contrast to the Hill coefficient of 1 for the cyanylation or acylation (by FAP) reactions. NAD^+ accelerates the reaction of the yeast enzyme with iodoacetic acid and decreases the rate with iodoacetamide (Table I), similar to the results with the rabbit muscle enzyme (MacQuarrie and Bernhard, 1971b).

These results are difficult to explain on any basis other than a highly specific ligand induced change. It has been postulated in the rabbit muscle enzyme (MacQuarrie and Bernhard, 1971b) that a specific charge effect may be operating between iodoacetate and NAD^+. Both NTCB and iodoacetate have free carboxylic acid groups which would have a negative charge at the pH's studied. Indeed, Fenselau (1970) has suggested that the role of NAD^+ as an active site director in the rabbit muscle enzyme is due to an ionic interaction. That the enhanced rate of iodoacetate and NTCB described here may at least be partially attributed to an ionic interaction is supported by the observation that the rate of modification of the apoenzyme by iodoacetate is decreased by adding salt (NaCl) while the higher modification rate of the holoenzyme is unaffected by the increased salt content of the medium (up to 0.3 M). However, the simple explanation that NAD^+ enhances these reactions by facilitation of the binding of the modifying reagent seems unlikely. In the absence of NAD^+, iodoacetate inactivation shows saturation kinetics with K_d of 7 (± 1) $\times 10^{-3}$ M (at $\mu = 0.12$ M and pH 8.5). NTCB, however, shows no such saturation kinetics. MacQuarrie and Bernhard (1971b) have demonstrated that the effect of NAD^+ on the iodoacetamide alkylation reaction of the rabbit muscle enzyme cannot be attributed to the small change in pK_a (about 0.1 unit) of the active site cysteine residue.[4]

NAD^+ has appreciably different effects in the yeast and muscle species of the furylacryloyl-enzyme. It decreases the rate of hydrolysis of the acylated yeast enzyme 17-fold (1 mM NAD^+), but increases the rate of hydrolysis of the acylated rabbit muscle enzyme (Malhotra and Bernhard, 1973). However, with both the rabbit muscle enzyme and the yeast enzyme the deacylation rate depends more strongly on the NAD^+ concentration (Hill coefficient >1) than does the acylation rate. Also, NAD^+ induces a red shift from λ_{max} 344 to 360 mm in the chromophoric acyl spectrum of the rabbit muscle enzyme (Malhotra and Bernhard, 1973) while no such shift is observed in the yeast enzyme ($\lambda_{max} \approx 350$ mM) even at 10 mM NAD^+.

Furthermore it is unlikely that the acylation rate enhancement brought about by NAD^+ for the yeast enzyme is simply an electrostatic effect. The pseudo-first-order acylation (by FAP) rate constants in the presence of NAD^+ (at approximately 95% saturation) are larger by 50–75-fold than the rate constants for acylation in the presence of the analog acetylpyridine adenine dinucleotide [at about 40% saturation (Stockell, 1959)].

Reactivity with Various Nucleophiles. To further clarify the role of NAD^+ in the deacylation of the phosphoglyceroylated enzyme, the enzyme-catalyzed oxidation of G3P was followed in the presence of 1 mM NAD^+. In the absence of any added nucleophile the progress curve for the production of NADH is biphasic: a rapid transient release of 3.5–4 mol of NADH/tetramer is observed followed by a linear steady-state production of NADH. Addition of 10 mM sulfate does not perturb the progress curve. In the

[4] The pH dependence for the alkylation of yeast GPD by iodoacetamide (in the absence of NAD^+) depends on an acidic group with $pK_a = 8.5$ in 0.1 M NaCl ($\mu = 0.12$) [$k_2^{lim} = 4.5(\pm 0.1) \times 10^4$ M^{-1} min^{-1}] (K. K. Yen, L. D. Byers, and D. E. Koshland, Jr., unpublished observations). The pH dependence for alkylation of the apo-rabbit muscle GPD by iodoacetamide depends on a group with $pK_a = 8.0$ ($k_2^{lim} = 1.4 \times 10^4$ M^{-1} min^{-1}, 0.1 M KCl) and the alkylation of the holoenzyme depends on a group with $pK_a = 8.1$ ($k_2^{lim} = 1.1 \times 10^3$ M^{-1} min^{-1}, 0.1 M KCl) (MacQuarrie and Bernhard, 1971b).

Table II: Relative Reactivities of Some Nucleophiles with Phosphoglyceroyl GPD.[a]

X	Concn (mM)	pK_a[2]	v_X/v_{H_2O}
H_2O		15.7	1
SO_4^{2-}	10	2.0	1
$HOAsO_3^{2-}$	1	7.0, 11.5	360
$CH_3PO_3^{2-}$	1	7.1	410
$HOPO_3^{2-}$	1	7.2, 12.3	4400
$HP_2O_7^{3-}$	10	6.6, 9.2	0.9
$B_4O_7^{2-}$	10	9.0	0.36

[a] Conditions: 1.17×10^{-8} M enzyme, 1 mM NAD^+, 0.75 mM D-G3P, 0.425 M bicine, and 0.425 mM EDTA (pH 8.5), 25°. All Na^+ salts. Velocities are initial values of NADH production (in the interval between 1 and 2 min after the addition of G3P) in the presence of yeast phosphoglycerate kinase (ca. 40 μg/ml), ADP (2×10^{-4} M), and Mg^{2+} (5 mM) to remove inhibition by the products 1,3-diphosphoglycerate and 1-methylphosphonato-3-phosphoglycerate. Under these conditions the rate-limiting step is deacylation of the acyl-enzyme (see text). [b] Thermodynamic value, from Jencks and Regenstein (1968).

Table III: Relationships between Half-of-the-Sites Activity in Enzyme Assay and Reagent Reactivity.

Reagent	Half-Site in Assay[a] Yeast GPD	Half-Site in Reactivity[b] Yeast GPD	Half-Site in Assay[a] Rabbit GPD
Mercurials			
p-Chloromercuribenzoate	Yes[c,d]		No[g,h]
Methylmercury	No[d]		
Acylating reagents			
β-(2-Furyl)acryloyl phosphate	Yes[c]	Yes[c]	Yes[i]
Acetyl phosphate	Yes[c]	Yes[c]	No[i]
p-Nitrophenyl esters	Yes[c]	No[c]	No[i]
Alkylating reagents			
NTCB	Yes	No	
Iodoacetate	Yes[c]	Yes[c]	No[k]
Iodoacetamide	Yes[c]	Yes[c]	No[l]
1-Fluoro-2,4-dinitrobenzene	Yes[c]	Yes[c]	Yes[k,m]
N-Ethylmaleamide	Yes[e]	Yes[e]	No[k]
Disulfides			
5,5′-Dithiobis(2-nitrobenzoate)	Yes[c]	No[f]	No[k]

[a] Inhibition of dehydrogenase activity at pH 8.5. [b] Reagent reactivity (at pH 8.5) is considered half-of-the-sites when the first two groups react at faster rates than the second two groups. [c] Stallcup and Koshland, 1973a. [d] Halsey, 1955. [e] Holland and Westhead, 1973. [f] von Ellenrieder et al., 1972. [g] Velick, 1953. [h] Smith and Schachman, 1971. [i] Malhotra and Bernhard, 1968. [j] Harris et al., 1963. [k] Levitzki, 1973; Levitzki, 1974. [l] MacQuarrie and Bernhard, 1971b. [m] Shaltiel and Sornia, 1969.

presence of arsenate a linear steady-state production of NADH is also observed but the rate is much faster than in the absence of arsenate. This rate is directly proportional to the arsenate concentration (from 1 to 20 mM), with k_2 = $1.3(\pm 0.06) \times 10^5$ M^{-1} min^{-1} (1 mM NAD^+–0.75 mM G3P).

When phosphate or methyl phosphate are substituted for arsenate the progress curve is nonlinear due to strong product inhibition (Smith and Velick, 1972). In the presence of phosphoglycerate kinase and MgADP (at concentrations which have no effect on the arsenolysis reaction) the inhibitory product is removed and initial velocities can be more easily determined. The observation that the steady-state rate depends on the nature and concentration of the nucleophiles is consistent with the rate-limiting step being the deacylation of the phosphoglyceroylated enzyme (i.e., NADH release must be greater than 10^4 min^{-1}).

The rate of reaction of the acylated yeast enzyme with various nucleophiles (at subsaturating concentrations) is shown in Table II. The rate of reaction of phosphate, the natural substrate for the enzyme, is the fastest of the nucleophiles tested and slower but significant rates are observed with arsenate or methyl phosphate. Water reacts more slowly with the enzyme. Tetraborate and pyrophosphate inhibit the hydrolysis reaction. Thus, the enzyme is designed to distinguish between water and its natural substrate, phosphate.

As indicated in Table I, the phosphorolysis and arsenolysis reactions are *slower* than the hydrolysis reactions of the acylated enzymes in the absence of NAD^+. [For example, deacylation of the phosphoglyceroylated or furoylacryloylated enzyme is independent of phosphate or arsenate concentrations (up to at least 20 mM), and equal to the hydrolysis rate, in the absence of NAD^+.]

If these figures are now contrasted with those of Table II an interesting feature of the NAD^+-induced change is revealed. In the *absence* of NAD^+ the rate of reactivity with water is faster than with phosphate. The NAD^+-induced conformational change increases both hydrolysis and phosphorolysis but accelerates the phosphorolysis reaction far more readily than the hydrolysis reaction. The NAD^+-induced conformational change is highly specific in nature; it does not simply destabilize an acyl-enzyme intermediate, but rather leads to a change which specifically favors the phosphorolysis reaction over the hydrolysis reaction.

Discussion

Nature of Probes and Conformational Changes. Chemical reactivity is one of the most sensitive probes of the conformational state of a residue and the use of different reagents, as described above, can explore the subtleties of conformational changes in a particularly effective way. The results dramatize the danger of making generalized conclusions in regard to the whole protein from the results of one probe. For example, the pseudo-first-order kinetics for the reaction of the enzyme with NTCB indicates that four cysteine-149 sulfhydryl residues react independently and noncooperatively. To deduce from this that the subunits generally act independently would clearly be erroneous since iodoacetamide shows half-of-the-sites reactivity with the same protein on alkylation of the same cysteine-149 residue. The possibility that the cyanylating agent is incapable of detecting conformational changes can be eliminated since the cyanylating agent is extremely unreactive toward the unmodified subunits of the dicarboxyamidomethylated enzyme.

The independence of the four sites in the cyanylation reaction does not exclude conformational changes which are transmitted across subunits. The enzymic activity of a monosubstituted yeast glyceraldehyde-3-phosphate dehydrogenase is only 40% of the native enzyme and the disubstituted protein has 0% of the activity. Quite clearly, then, cyanylation of the cysteine residue induces conformational changes or prevents subsequent conformational changes induced in the catalytic reaction. By one probe, i.e., the effect of NTCB on its own reactivity, it might be concluded that

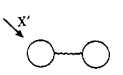
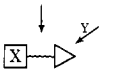
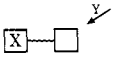
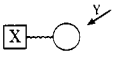
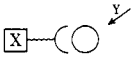
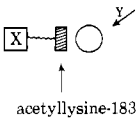
cyanylation has no effect on neighboring subunits, but by another (the catalytic activity), quite clearly there is an effect on the transmission mechanism. The effect of cyanylation is not revealed by one probe alone.

It might be asked, "what are the special properties of these probes which alter their sensitivity?" The charge or structure of the reacting reagent must have some influence and the difference in reactivity between iodoacetic acid and iodoacetamide in reactions with GPD are an indication of this type of change. The binding of a substrate with the opposite charge to a reagent can obviously attract the reagent electrostatically. Also, alteration of the surface of the protein can exclude a more bulky reagent. On the other hand, an examination of Table III shows no single pattern separating the reagents giving half-of-the-sites reactivity (toward reagents) from those which give all-of-the-sites reactivity. Those reagents which are increased in the reactivity rate by NAD^+ (e.g., acylation by FAP and alkylation by NTCB and iodoacetate) are not easily distinguished structurally, from those which are decreased [e.g., acylation by *p*-nitrophenyl acetate (Park et al., 1961) and alkylation by iodoacetamide]. Quite obviously, there is a subtle relationship between the reactivity of the reagent and the change in the structure at the active site. Possibly in some reactions a pattern will develop as to these structures and to the reactivity changes. For example, Schlessinger and Levitzki (1974) have suggested that in the rabbit muscle enzyme the adenine position of the NAD^+ is important in the half-of-the-sites reactivity.

These results reveal the usefulness of reactivities as compared to physical tools for the recording of the conformational state of reactive residues. The usefulness of fluorescence, nuclear magnetic resonance (NMR), spin-label, etc., probes are clear and beyond dispute. Many studies have indicated that the orientation of substrates at active sites play an important part in their reactivity (Koshland et al., 1971). This indicates that probes which utilize chemical reactivity will be particularly sensitive. Subtle changes in conformation which cannot be said to cause a residue to be "exposed" or "buried" may still affect the orientation of a group at its active site or may alter neighboring groups so the reagent can only approach from a limited direction. If the reactivity is sensitive to this kind of orientation or to a slight change in the steric interactions during reaction, then this probe may delineate what more gross conformational probes will fail to detect.

The Nature of Induced Conformational Changes. In Table IV the induced conformational changes in yeast GPD are categorized with respect to their effects on some subsequent reactions. The functional effects of modification of one subunit on the other subunits depend on the nature of the modifier and the probe used for its detection. Thus, modification of one subunit can result in a conformational change in an adjacent subunit resulting in either a decreased, increased, or unaltered catalytic activity (e.g., alkylation by iodoacetamide, NAD^+ binding, and NADH binding, respectively). In many cases the effect on catalytic activity is parallelled by an equivalent effect on reagent reactivity (first three examples in Table IV). However, a modifier can also induce (or prevent) a subsequent conformational change in which reactivity toward modifiers is not parallelled by the catalytic reactivity change. Thus, cyanylation of one subunit has no effect on the sulfhydryl reactivity (toward NTCB) on the adjacent subunits but decreases catalytic activity. The observed decrease in catalytic activity of the unmodified subunit indicates either (a) a subtle induced conformation change in that subunit (detectable by the catalytic activity probe but not NTCB) or (b) a modification which blocks a conformational change essential for catalytic activity. Communication between subunits can indeed be modified as evidenced by the alkylation of GPD acetylated at lysine-183. Carboxymethylation of the active site cysteine-149 in the tetraacetylated enzyme proceeds independently at all four sites in contrast to the half-of-the-sites reactivity of the unacetylated enzyme. Thus, acetylation blocks transmission between subunits of the conformational change induced by carboxymethylation.

Table IV: Intersubunit Communication.[a]

Effect of Modification of First Subunit on Reactivity of Unmodified Subunit		Modification Reagent X	Probe Y
Decreased[b]		$-CH_2CO_2^-$	$ICH_2CO_2^-$ catalytic activity
Increased[c]		NAD^+	NAD^+ binding (to 3rd site) catalytic activity
Unaltered[d]		NADH	NADH binding catalytic activity
Unaltered toward some probes; decreased toward others		$-CN$	2-Nitro-5-thiocyanobenzoate (increased reactivity) catalytic activity (decreased)
Eliminates communication[e]		$-CH_2CO_2^-$	$ICH_2CO_2^-$

[a] Intersubunit communication in GPD as evidenced by various probes (Y) of the partially modified enzyme. For simplicity only the dimeric component of the protein is illustrated. [b] Stallcup and Koshland, 1973a. [c] Cook and Koshland, 1970; Kirschner et al., 1971; Sloan and Velick, 1973. [d] von Ellenrieder et al., 1972. [e] Stallcup and Koshland, 1973b.

The examples given illustrate several basic principles of conformational changes. (1) The ligand-induced changes can alter some parts of a protein without disturbing others both within one subunit and between subunits. (2) The induced changes can deactivate some sites (inhibition or negative cooperativity) while activating others (activation or positive cooperativity). (3) The changes can be activating as revealed by one probe and neutral or inhibiting as revealed by another. (4) The changes can be induced by noncovalent binding or by covalent modification. (5) Neither the protein as a whole nor the subunit can be considered as a unit since the induced changes are focussed to be transmitted to specific parts of the protein.

The extensive findings of negative cooperativity, mixed cooperativity, and proteins in which one substrate induces positive cooperativity and another negative cooperativity indicate that the multistate nature of conformational changes shown in Table IV is probably a universal one. It also illustrates the danger of deducing the effect of a molecule from one probe or one cooperativity pattern. Thus the placing of reporter groups such as spin-labels in a protein could reveal quite different patterns depending on where they are

placed. One site would reveal no cooperativity whereas another site would indicate cooperativity. These conflicting messages would be confusing if one assumed a concerted model in which all sites change concurrently, but is readily understandable if the protein programming is more focussed and more individualized.

Biological Advantages. A simplistic picture such as "NAD^+ stabilizing modified protein" or "NAD^+ destabilizing the apoprotein" is clearly unable to explain these results. NAD^+ accelerates certain reactions and decelerates others. It destabilizes the acyl-enzyme toward some reactions and stabilizes it toward others. A general explanation of this NAD^+ effect can be obtained by restating the observations in terms of transition-state theory. When NAD^+ accelerates the reaction rates it must bind more tightly to the enzyme in the transition state than in the ground state. For reactions in which different transition-state structures are expected (e.g., phosphorolysis vs. hydrolysis of the acyl-enzyme) it is reasonable to expect that NAD^+ can induce and stabilize one structure (e.g., a protein complementary to the transition state for the physiological phosphorolysis reaction) and destabilize the other.

The specificity, most notably in reactions of hydrolysis and phosphorolysis, has important biological significance. The function of glyceraldehyde-3-phosphate dehydrogenase is to produce a high energy intermediate, 1,3-diphosphoglycerate. Any hydrolysis reaction causes loss of potential ATP molecules and is a waste of energy of the glycolytic pathway (derived, specifically from the oxidation of glyceraldehyde 3-phosphate). NAD^+ accelerates the overall reaction, but does so in a very specific manner by increasing reactivity toward the phosphate nucleophile at the expense of the water nucleophile. The conformational change is sensitive to the specific substrates, NAD^+ and phosphate, and does not give the same reaction to other small molecules. Thus, the action of the effector on the acyl transfer reaction depends on the acyl group (phosphoglyceroyl or furylacryloyl), the structure of the effector (NAD^+ or its analogs), and the nature of the nucleophile (phosphate, phosphate analogs, or water). This means that the conformational change is focussed in the direction of creating a protein state which is more reactive toward its physiological substrates and less reactive toward extraneous competing compounds in the medium.

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Effects of pH and Thiols on the Kinetics of Yeast Glyoxalase I. An Evaluation of the Random Pathway Mechanism†

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ABSTRACT: The disproportionation of α-ketoaldehydes, catalyzed by yeast glyoxalase I, has been reported to involve a random pathway mechanism where one branch utilizes the hemimercaptal of glutathione and the α-ketoaldehyde in a one-substrate pathway, and the other branch utilizes first glutathione and then the α-ketoaldehyde in an ordered two-substrate pathway. The relative importance of the two pathways has been evaluated at 5° in the pH range 3–7, using methylglyoxal and phenylglyoxal as representive aliphatic and aromatic α-ketoaldehydes, by comparing initial rates of hemimercaptal formation in the absence of enzyme with initial rates of product formation in the presence of high enzyme concentrations. If the enzyme is not added last, the initial rates of product formation are the same as the initial rates of adduct formation even under conditions where it could be shown that dehydration of the hydrated α-ketoaldehyde is not entirely rate determining. If the enzyme is added after hemimercaptal formation, there is a "burst" of product formation equivalent to the amount of hemimercaptal, followed by a slower reaction, consistent with the one-substrate pathway. Additional support for this pathway was obtained from a study of the effects of added thiol reagents on the "burst" kinetics. The broad specificity of yeast glyoxalase I for both aliphatic and aromatic α-ketoaldehydes, reflected in V_{max} values which are insensitive to the nature of the α-ketoaldehyde, drops abruptly if the side chain of the α-ketoaldehyde is sterically crowded. The hemimercaptal of *tert*-butylglyoxal has a V_{max} 300-fold smaller than V_{max} for methylglyoxal; 2,4,6-trimethylphenylglyoxal is essentially inactive as a substrate even though the closely related compound 2,4-dimethylphenylglyoxal is a normal substrate. Analysis of the V_{max} and K_m (or K_i) values of these α-ketoaldehydes suggests that sterically crowded side chains affect both enzyme–substrate formation and the catalytic reaction.

The glyoxalase system catalyzes the disproportionation of α-ketoaldehydes, such as methylglyoxal, into α-hydroxycarboxylic acids. Two enzymic reactions take place: the first, catalyzed by glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5), requires glutathione as cofactor and involves the conversion of an α-ketoaldehyde into a thiol ester of glutathione and the corresponding α-hydroxycarboxylic acid; the second reaction, catalyzed by glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), is the hydrolysis of the thiol ester to regenerate glutathione and liberate a free α-hydroxycarboxylic acid. Both a two-substrate mechanism (Kermack and Matheson, 1957) involving methylglyoxal (M) and glutathione (G) as substrates (I) and a one-substrate mechanism (Cliffe and Waley, 1961) involving the hemimercaptal (A) of methylglyoxal and glutathione as substrate (II) have been proposed for glyoxalase I. The one-substrate mechanism has received some additional experimental support (Davis and Williams, 1969).

$$v = \frac{V[M][G]}{K + K_m^G[M] + K_m^M[G] + [M][G]} \quad (1)$$

$$v = V[A]/(K_m^A + [A]) \quad (2)$$

Recently, Mannervik and coworkers carried out a detailed steady-state kinetic analysis of both yeast and porcine erythrocyte glyoxalase I using nonlinear regression methods to select the best of a number of mathematical models for glyoxalase I (Mannervik et al., 1973, 1974). Under most concentration conditions, the steady-state data can be fitted by either a one-substrate mechanism

$$v = \frac{V[A]}{K_m^A[1 + ([G]/K_i)] + [A]} \quad (3a)$$

with glutathione as a competitive inhibitor of the hemimercaptal or by a two-substrate ordered mechanism

$$v = \frac{V[M][G]}{K + K_m^M[G] + [M][G]} \quad (3b)$$

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