Half-of-the-Sites Reactivity of Bovine Liver Uridine Diphosphoglucose Dehydrogenase toward Iodoacetate and Iodoacetamide†

James S. Franzen,* Raymond Ishman, and David S. Feingold‡

ABSTRACT: The reaction of bovine liver uridine diphosphoglucose dehydrogenase (UDPGDH) with iodoacetate and iodoacetamide has been investigated in terms of the stoichiometry and kinetics of alkylation, the kinetics of loss of enzyme activity, and the influence of enzyme substrates and inhibitors on these processes. It was observed that iodoacetate reacted exclusively with the catalytic site thiol groups in a biphasic manner. Three of the six subunits are carboxymethylated rapidly, and the other three react at a rate which is an order of magnitude lower. The loss of enzyme activity is greater than the degree of incorporation of iodoacetate, showing that carboxymethylation of one subunit influences the catalytic activity of its neighbor. The catalytic site thiols are protected by UDP-glucose (UDPG) and UDP-xylose (UDPX) but not by NAD^+ or NADH. The reaction with iodoacetamide also shows a biphasic initial period; however, iodoacetamide reacts elsewhere on the enzyme as well as at the catalytic site. UDPG and UDPX protect the catalytic site thiols against carboxamidomethylation by iodoacetamide. NAD^+ and NADH increase the reactivity of the catalytic site thiols with iodoacetamide but decrease the reactivity of the noncatalytic site thiols. The results suggest that UDPGDH subunits are functionally related in a pairwise manner, and the topology of the active site determines that the glucose moiety of bound UDPG is flanked on one side by the essential enzyme thiol group and on the other by NAD^+.

Uridine-diphosphoglucose dehydrogenase, UDPGDH[1] (EC 1.1.1.22), from bovine liver has been shown to have a thiol group at the active site that directly participates in the chemistry of the second stage of the catalytic four-electron transfer process (Ridley et al., 1975). The reactivity toward DTNB of this and the 11 other thiol groups on each subunit of this hexameric enzyme has been studied in some detail (Gainey et al., 1972; Uram et al., 1972). The reaction of UDPGDH with DTNB, for example, takes place preferentially at the catalytic sites of the enzyme as is evidenced by the linear loss of enzymatic activity with titration of the first six thiol groups (Gainey et al., 1972). The early reaction with DTNB is of further interest because of the complex kinetics it displays. According to Gainey et al. the reaction is characterized by an initial transient corresponding to the titration of two thiol groups per hexamer, followed by a series of slower stages, some of which occur only with the denatured enzyme. The stopped-flow kinetics records of these authors appear to indicate that NAD^+ greatly augments while NADH inhibits the initial transient, "hidden burst". UDPG and UDPX serve as strong inhibitors of the DTNB reaction.

The kinetics of the DTNB reaction just mentioned, in which one-third of a site per subunit reacts rapidly, implicates site–site interactions in UDPGDH. Such interactions may reflect differential functions of neighboring subunits in the process of enzymatic catalysis. In this regard the recent report of Ordman and Kirkwood proposes the consecutive participation of neighboring subunits in the two stages of oxidation of UDPG (Ordman and Kirkwood, 1976). The structural attributes of the enzyme, that would cause the catalysis to proceed by the suggested flip-flop scheme, could also account for fraction-of-the-sites behavior such as active site thiol group reactivity. Evidence for half-of-the-sites reactivity in UDPGDH comes from binding studies with the substrates, UDPG and NAD. Equilibrium dialysis experiments have shown that the enzyme is saturated with UDPG when three of the six subunits are occupied by this ligand (Franzen et al., 1973). The companion substrate, NAD^+, also appears to be able to bind only to three subunits, as determined by equilibrium dialysis and differential fluorescence (Franzen et al., 1973) and by the Hummel–Dryer gel filtration technique (ainey and Phelps, 1974). However, the weak affinity for NAD^+ makes it difficult to be sure that saturation was really achieved in these investigations.

In light of these considerations, we have examined the reactions of iodoacetate and iodoacetamide with UDPGDH. These reagents have been very useful in elucidating the half-of-the-sites behavior of glyceraldehyde-3-phosphate dehydrogenase, an enzyme which like UDPGDH utilizes a thiol in the oxidation of an aldehyde to an acid (Mac Quarrie and Bernhard, 1971; Stallcup and Koshland, 1972). We have observed that iodoacetate and iodoacetamide react readily with UDPGDH, iodoacetamide being strongly directed to the catalytic site and iodoacetate being exclusively so directed. There are substantial interactions between derivatized catalytic and nonderivatized catalytic sites and these sites primarily influence each other in pairs. With iodoacetamide, which can also react with thiols elsewhere on the enzyme, it is possible to observe interactions between the catalytic sites and these other sites under certain conditions.

Experimental Procedures

Materials. [^{3}H]Iodoacetic acid (206.6 mCi/mmol) and [^{14}C]iodoacetamide (9.35 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). These materials were recrystallized prior to use along with their nonradioactive

† From the Department of Life Sciences, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received July 22, 1976.* This work was supported by grants from the National Institutes of Health (AM 15332 and GM 19649).

‡ Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261.

[1] Abbreviations used are: UDP (GDH, G, GA, X), uridine diphospho (glucose dehydrogenase, glucose, glucuronic acid, xylose); DTNB, 5,5′-dithiobis(2-nitrobenzoate); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

TABLE I: Quantitation of Procedure for Measuring Incorporation of Iodoacetate (IAc) and Iodoacetamide (IAm). Equivalence of Thiol Content and Extent of Alkylation.[a]

Sample	Thiols per Subunit by DTNB Reaction	Extent of Alkylation (alkyl groups/subunit)	
		With IAc	With IAm
1	10.3	10.0	
2	10.2		9.4

[a] The DTNB and alkylation reactions were performed in 6 M urea, the denaturing agent being added last. DTNB was present at a concentration of 150 μM and the enzyme at 5 to 6 μN. For the alkylation reaction, alkylating reagents were 1.3 mM and the enzyme at 5 to 6 μN.

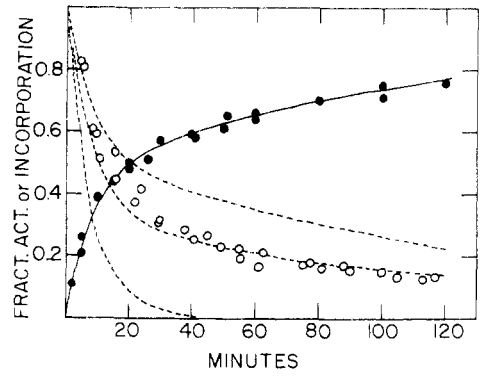


FIGURE 1: Time course of carboxymethylation and activity loss. Combined data from two experiments on the reaction of iodoacetate, 812 μM, with UDPGDH at concentrations of 7.0 and 12.9 μN. The line through the incorporation data, closed circles, is drawn according to eq 1, for $k_1 = 2.00 \times 10^{-3}\ s^{-1}$ and $k_2 = 1.17 \times 10^{-4}\ s^{-1}$. The dashed line decay curves are drawn according to eq 4 for ϕ values of 1.0, top curve; 0.6, curve through observed inactivation data; and 0.0, bottom curve. Initial enzyme specific activity was 1.65 units/mg.

counterparts from *n*-hexane and chloroform, respectively. The enzyme was prepared essentially according to the procedure of Zalitas and Feingold with slight modification (Zalitas and Feingold, 1969). For example, the initial extraction was carried out with a proteinase inhibitor, phenylmethylsulfonyl fluoride, present at a concentration of 5 mg/l., and Sephadex G-200 was replaced by Ultrogel, type AcA 34, obtained from LKB. Enzyme preparations used in this study ranged in specific activity from 1.3 to 2.0 units per mg when assayed according to conditions established earlier (Zalitas and Feingold, 1969). Note that units as defined here refer to micromoles of UDPG oxidized per minute.

Methods. The enzyme was stored at a concentration of approximately 1% at pH 5.6 in 0.2 M acetate buffer, 0.1 M in β-mercaptoethanol, and 0.002 M in EDTA. Reactions with iodoacetate or iodoacetamide were carried out at pH 8.0 in 0.05 M Tris buffer, 0.002 M in EDTA, at 30 °C in darkened tubes. Just prior to reaction with iodoacetate or iodoacetamide, a 0.080-ml aliquot of stock enzyme was passed through a G-25 Sephadex column (6 cm × 0.6 cm) previously equilibrated with the Tris buffer. The enzyme-containing fractions of the void volume were combined and the concentration of enzyme was determined by the absorbance at 277 nm ($A_{1cm,277}^{1mg/ml} = 0.98$). To this solution, a solution of the alkylating reagent, containing substrate or inhibitor ligands, as indicated, was added to start the reaction. Aliquots of the reaction mixture were removed at various times to assay either for incorporation or enzymatic activity.

For the assay of incorporation of iodoacetate or iodoacetamide, reaction mixture aliquots containing about 25 μg of enzyme were withdrawn and added to 1 ml of cold 7% Cl_3CCOOH–5% phosphotungstic acid. The solutions were vigorously mixed and allowed to stand for 15 min in ice. The solutions were then filtered through glass fiber filters (Whatman, GF-C), and the filters were then washed four times with 2 ml of 7% Cl_3CCOOH–5% phosphotungstic acid, and four times with ether. The filters were allowed to dry and then transferred to counting vials. To each of these vials was added 0.5 ml of 0.5 N quaternary ammonium hydroxide in toluene (Soluene 100, Packard Inst., Co.), and the vials were allowed to stand for 30 min. Ten milliliters of scintillation fluid was next added and the samples were counted. Blank samples were prepared by precipitating an equivalent amount of protein with Cl_3CCOOH–phosphotungstate and then adding radioactive reagent and filtering and working up as above. Practical specific activities of the alkylating agents were determined by precipitating and filtering the usual amount of enzyme and then adding an aliquot of the radioactive reagent solution to the dry filter. After air drying, the filter was counted in the usual way. All incorporations are reported on the basis of moles of alkyl group per mole of subunit of enzyme. The subunit molecular weight is 52 000 (Uram et al., 1972; Huang et al., 1971; Gainey et al., 1972). All enzyme concentrations are reported as μN, i.e., as the number of micromoles of subunits per liter.

For the assay of enzyme activity during the alkylation reaction, 10 μl of reaction mixture was withdrawn and plunged into 1.0 ml of assay medium at 30 °C. Assay medium was comprised of 0.1 M glycylglycine buffer, pH 8.5, 1.0 mM in NAD, and 1.0 mM in UDPG. The 100-fold dilution served both to stop the alkylation reaction and remove the inhibition of any enzyme inhibitors present in the alkylation reaction mixture in certain experiments. The inhibitory effects of such inhibitors were not only reduced by dilution but suppressed by the saturating concentrations of substrates.

Results

Carboxymethylation, Relation between Incorporation and Activity. Initial experiments with iodoacetate indicated that there was a rapid loss of enzyme activity, but very low incorporation of carboxymethyl groups, e.g., on the order of 0.2 to 0.5 carboxymethyl groups per subunit. Apparently the protein precipitations performed in these experiments with 5 or 10% Cl_3CCOOH were not quantitative. Complete retention of enzyme protein on the filters was achieved by supplementing the precipitating solution with 5% phosphotungstic acid. Under these conditions it was possible to demonstrate stoichiometric blocking of enzyme thiols by iodoacetate and iodoacetamide when the enzyme was subjected to denaturation as seen in Table I. The equivalence of the DTNB and alkyl group incorporation results suggests that under these conditions the reagents were reacting only at cysteine residues and not elsewhere in addition. This is so providing the presence of 6 M urea in the sample aliquot did not lower the retention of protein by the filter. Control experiments showed that the effectiveness of the filtration procedure was unchanged by the presence of denaturing amounts of urea in the reaction mix.

The kinetics of carboxymethylation of UDP-glucose dehydrogenase are presented in Figure 1. The significant features of this reaction are: (1) that a total of only one sulfhydryl group

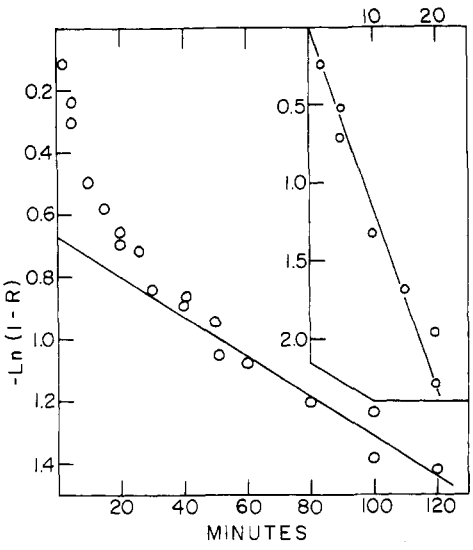


FIGURE 2: Logarithmic presentation of carboxymethylation reaction. Apparent pseudo-first-order rate constant, k_2, obtained from the line through the points at longer times is 1.06×10^{-4} s^{-1}. The inset shows a plot of $-\ln(1 - 2R_1)$ vs. time, where R_1 represents the incorporation at the fast "half site". Values for R_1 are obtained by correcting early time R values for incorporation at the slower sites, using the extrapolated early time region of the line in the $-\ln(1 - R)$ vs. time plot.

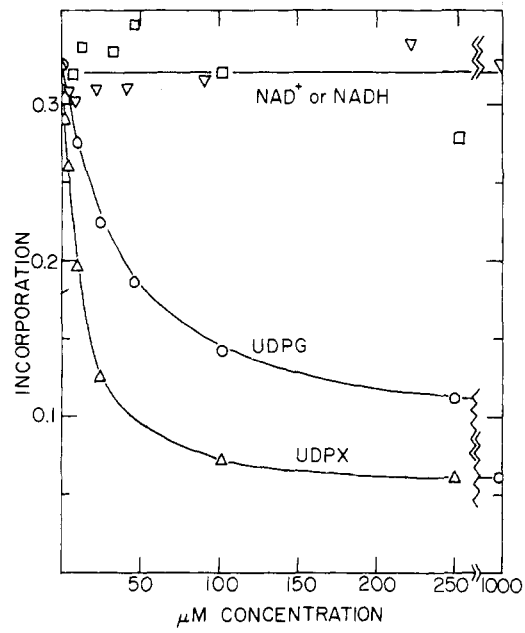


FIGURE 3: Effect of varying concentrations of UDPGDH substrates and inhibitors on carboxymethylation. Reactions were carried out for 10 min at an enzyme concentration of 14.8 μN and an iodoacetate concentration of 1390 μM.

per subunit is blocked; (2) that activity loss accompanies carboxymethylation in a similar biphasic manner but the activity loss is greater than carboxymethylation; and (3) that the reaction is strongly biphasic, one half-site per subunit reacts an order of magnitude more rapidly than the other half-site. Overnight reaction led to the incorporation of no more than one carboxymethyl group per subunit.

Figure 2 presents the data of Figure 1 in logarithmic form and clearly shows the biphasic nature of these data. In Figure 2 and eq 1-3, R is the number of moles of thiol derivatized per mole of subunit. From the slope of the function at longer times we find that a pseudo-first-order rate constant of 1.06×10^{-4} s^{-1} characterizes the second stage of the reaction and, from the corrected logarithmic plot of early time data, Figure 2 inset, a pseudo-first-order rate constant of 1.93×10^{-3} s^{-1} characterizes the first stage of the reaction. The solid lines of Figure 1 are generated for the model in which the reactive sulfhydryl groups, one per subunit, are assumed to be equally reactive initially, but influence each other in a pairwise manner such that, if one member of a pair is blocked, the rate constant for reaction with the other is changed. The rate of incorporation in this case is given by eq 1

$$R = 1 - e^{-k_1 t} - \frac{k_1}{2(k_2 - k_1)}(e^{-k_1 t} - e^{-k_2 t}) \qquad (1)$$

where k_1 is the pseudo-first-order rate constant characterizing all active site thiols initially, and k_2 is the corresponding rate constant for sites whose partners have already been derivatized (Frost and Pearson, 1953). For the data of Figure 1, observe that an acceptable fit is achieved with $k_1 = 2.00 \times 10^{-3}$ s^{-1} and $k_2 = 1.17 \times 10^{-4}$ s^{-1}. The values of the rate constants for the two stages of the reaction obtained from Figure 2 served as initial values for fitting Figure 1 data by eq 1. Of course, there is no a priori reason for choosing a site-site interaction model over a model in which there are two intrinsically different kinds of subunits. In the latter case the proper rate expression is given by eq 2. In this case, a curve which is virtually indistinguishable from the one drawn in Figure 1 is obtained by setting $k_1 = 1.83 \times 10^{-3}$ s^{-1} and $k_2 = 1.00 \times 10^{-4}$ s^{-1}

$$R = 1 - \frac{e^{-k_1 t} + e^{-k_2 t}}{2} \qquad (2)$$

Clearly no decision can be made on the basis of the incorporation kinetics as to which model represents the enzyme. The uppermost dashed line of Figure 1 portrays the loss of enzyme activity to be expected from blocking the catalytic sites according to the interaction model of eq 1. It is obvious that the activity loss exceeds the incorporation, particularly in the later stages of the reaction.

Carboxymethylation, Effects of Substrates and Inhibitors. The effects of the substrates and of the enzyme inhibitors, NADH and UDPX, on the incorporation are shown in Figure 3. The amount of incorporation at 10 min is seen to be unaffected by NAD^+ and NADH over a wide range of concentrations encompassing the entire saturation isotherms for these ligands. On the other hand UDPG and UDPX show marked inhibition of alkylation by iodoacetate, UDPX is known to bind to the enzyme with greater affinity than UDPG, and this preference is also exhibited in the effect on alkylation. The concentrations of UDPG and UDPX producing half-maximal inhibition of carboxymethylation are 55 and 12 μM, respectively. For purposes of comparison, the K_m value of UDPG is 13 μM (Zalitas and Feingold, 1969) and the K_I for UDPX is 4 μM (Neufeld and Hall, 1965). The inhibition of alkylation by UDPG confirms the likely proposition that iodoacetate attacks UDPGDH at the catalytic site.

The kinetics of carboxymethylation and the accompanying loss of activity in the presence of the substrates and the inhibitors, NADH and UDPX, are presented in Figure 4. The strong inhibitor of the enzyme, UDPX, acts to protect completely the enzyme against inactivation by iodoacetate, though some slow incorporation does seem to occur, presumably at other sites. Reaction at sites other than the active site is prohibited if the active site is carboxymethylated, but apparently, if it is not carboxymethylated, other sites can slowly be attacked. Rate constants corresponding to eq 1 were not evalu-

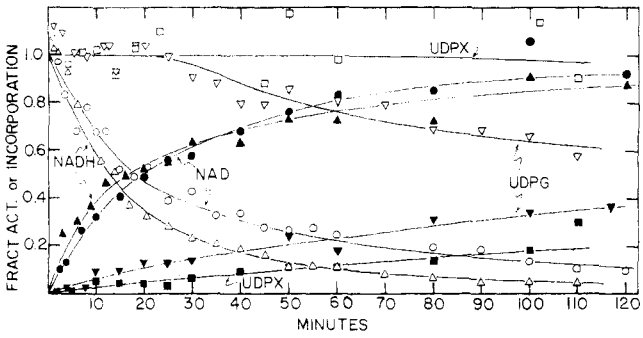


FIGURE 4: Effect of UDPGH substrates and inhibitors on time course of carboxymethylation. Incorporations are represented by filled symbols, and fractional activity losses by open symbols. Iodoacetate concentrations were 792 μM and enzyme concentrations ranged from 5 to 14 μN (incorporation kinetics in terms of R are independent of enzyme concentration for pseudo-first-order conditions). NAD, 1000 μM (●—○); NADH, 100 μM (▲—△); UDPG, 1000 μM (▼—▽); UDPX, 100 μM (■—□).

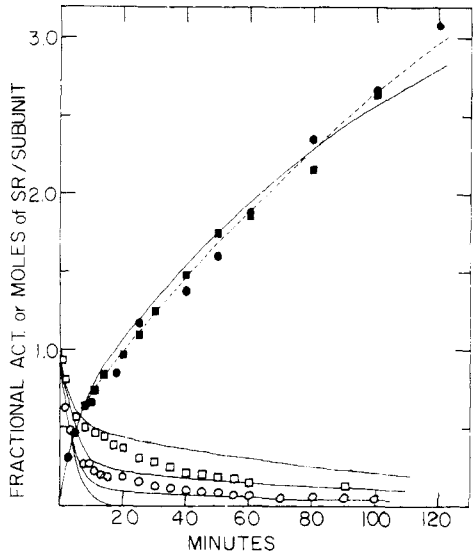


FIGURE 5: Time course of carboxamidomethylation and activity loss. The iodoacetamide concentration was 1584 μM and the enzyme concentrations for two different experiments were 10.3 μM, sp act. = 1.65 units/mg (●, ○); and 9.0 μM, sp act. = 1.28 units/mg (■, □). The solid line for incorporation is drawn using eq 3 and $k_1 = 4.5 \times 10^{-3}$ s^{-1}, $k_2 = 1.5 \times 10^{-4}$ s^{-1}, $n = 3$, and $k_3 = 1.5 \times 10^{-4}$ s^{-1}. The dashed line is for the same values of k_1 and k_2, but with $n = 6$ and $k_3 = 5.12 \times 10^{-5}$. The activity decay curves are drawn according to eq 3, and in descending order are for ϕ = 1.0, 0.5, 0.2, and 0.

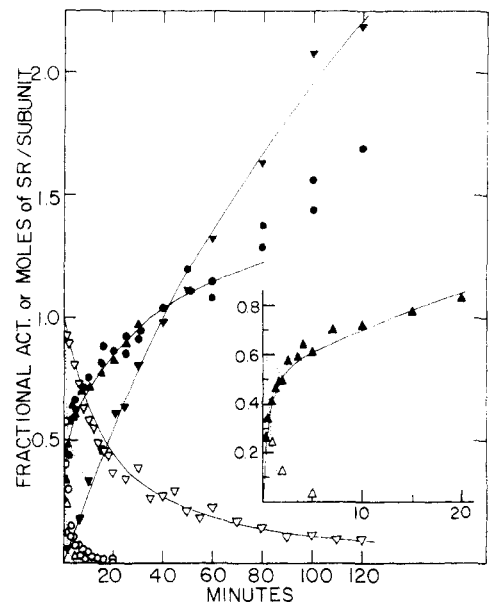


FIGURE 6: Effect of UDPG, NAD, and NADH carboxamidomethylation and activity loss. Closed symbols represent incorporation and open symbols represent activity loss. Iodoacetamide concentration was 1580 μM and enzyme concentration was 9.0 μN. UDPG present at 862 μM (▼, ▽); NAD present at 750 μM (●, ○); NADH present at 100 μM (▲, △). Incorporation line is drawn according to eq 3 for $k_1 = 2.6 \times 10^{-2}$ s^{-1}, $k_2 = 7.5 \times 10^{-2}$ s^{-1}, $k_3 = 8.33 \times 10^{-6}$, and $n = 6$. The dotted decay curves, drawn according to eq 4, employ the same values of k_1 and k_2 and ϕ values of 1.0 (upper curve) and 0.0 (lower curve). The inset displays the reaction data in the presence of NADH on an expanded time scale.

ated for reactions in the presence of UDPG, or UDPX, since the extent of inhibition probably involves the equilibrium between ligand-bound and ligand-free enzyme, necessitating a more complicated rate expression. Interestingly, even UDP, at 3 mM concentration acts as an inhibitor like UDPG or UDPX of the reaction with iodoacetate (data not shown). Also from Figure 4 we see that the addition of saturating amounts of the substrate NAD^+ has little effect on the kinetics of the incorporation reaction. Likewise NADH, a strong inhibitor of the enzyme, has little effect on the incorporation of carboxymethyl groups.

Carboxamidomethylation, Relation between Incorporation and Activity. Reaction of the enzyme with iodoacetamide yields results similar to but yet quite distinct from those with iodoacetate. As depicted in Figure 5, there is an initial fast incorporation reaction involving one half-site per subunit, followed by slower incorporations at the other half site and at still other sites. The activity loss initially coresponds to the fast incorporation and then proceeds at a much lower rate. If we assume that the behavior of the enzyme toward iodoacetamide is like its behavior toward iodoacetate except that n additional thiol groups are reactive and each of these n groups reacts with roughly the same rate constant, then eq 3

$$R = 1 - e^{-k_1 t} - \frac{k_1}{2(k_2 - k_1)}(e^{-k_1 t} - e^{-k_2 t}) + n(1 - e^{-k_3 t}) \quad (3)$$

represents the incorporation kinetics. For the data of Figure 5, acceptable fits are obtained for $k_1 = 4.5 \times 10^{-3}$ s^{-1} and $k_2 = 1.5 \times 10^{-4}$ and n and k_3 equal to 3 and 1.5×10^{-4} s^{-1}, or with n and k_3 equal to 6 and 5.12×10^{-5} s^{-1}. Overnight reaction with iodoacetamide leads to the incorporation of 4.5 carboxamidomethyl groups per subunit. It is not important for the purposes of this study to settle on the exact number of noncatalytic center thiols. The incorporation at noncatalytic site thiol groups may require a more complicated model than that described by eq 3; however, the simple model presented here adequately represents the data. The decay kinetics for inactivation resulting only from carboxamidomethylation of the active site thiol groups is given by the uppermost decay curve of Figure 5. As with carboxymethylation, it is observed that inactivation of enzyme activity in the later stages of the reaction with iodoacetamide exceeds the calculated extent of incorporation at the catalytic site of each subunit.

Carboxamidomethylation, Effects of Substrates and Inhibitors. The effects of substrates on the reaction with iodoacetamide are seen in Figure 6. As with the iodoacetate reaction, UDPG impedes the reaction with iodoacetamide at the catalytic site as is evidenced by the relative protection of enzymatic activity and slower initial rate of incorporation of

reagent. Inhibition of incorporation of iodoacetamide by UDPG is not as strong as the inhibition of incorporation of iodoacetate by UDPG. This may reflect the influence of carboxamidomethyl groups attached to noncatalytic site thiol groups on the reactivity of catalytic site thiol groups and/or on the binding of UDPG at the catalytic site. In the presence of UDPG, the enzyme is probably preferentially derivatized at noncatalytic site thiols. Blockage of these thiols may activate the catalytic site thiol, or may reduce the affinity of UDPG for the catalytic site. While UDPG dampens the reactivity of the active site thiol group toward iodoacetamide, NAD^+ enhances the reactivity of this group. Following the very rapid initial alkylation reaction, there is a much reduced second phase reaction both at the remaining catalytic half-site thiol groups and at secondary sites. The loss of enzymatic activity is extremely fast and is greater than the incorporation level, e.g., at an incorporation of 0.75 carboxamidomethl group the enzyme activity has dropped to 1-2% of its initial value. The reaction at the first half-site thiol is also very rapid in the presence of NADH. The inset of Figure 6 shows that by 2 min this site is completely alkylated and the enzymatic activity of the protein has fallen to 12% of its starting level. If eq 3 is used to describe the incorporation data, a reasonable fit can be obtained for the data out to 30 min if $k_1 = 2.6 \times 10^{-2}\ s^{-1}$, $k_2 = 7.5 \times 10^{-3}\ s^{-1}$, $k_3 = 8.33 \times 10^{-6}\ s^{-1}$, and $n = 6$. The reaction is not describable by eq 3 for longer periods of time. Thus the pyridine nucleotide substrate and product appear to enhance k_1 and k_2 five- to sixfold, while reducing k_3 by the same factor.

The enzyme inhibitor UDPX markedly reduces the rate of reaction of the active site thiol group with iodoacetamide as shown in Figure 7B. Although alkylation proceeds at a moderate rate in the presence of UDPX, the activity of the enzyme does not decrease until the overall incorporation has reached a level of about 0.3 carboxamidomethyl groups per subunit. The catalytic site thiol groups then appear to become exposed, possibly due to weakened binding for UDPGA and UDPX.

Combinations of inhibitors produce different responses depending on the nature of the combination; for example in Figure 7B, it is observed that UDPX and NADH together produce essentially the same effect as UDPX alone on the reaction with iodoacetamide. On the other hand, the system with UDP and NADH in combination yields results (Figure 7A) which are intermediate between the effects of either of these ligands by itself. Thus, UDPX effectively can block the activating effect that NADH imposes on the catalytically important sulfhydryl group. On the other hand, though UDP can by itself inhibit alkylation, it cannot fully mask the activating influence of NADH.

Discussion

The primary observation of this work is that the catalytic sites of UDPGDH exhibit biphasic kinetics with respect to reactivity toward the alkylating agents employed, as seen from Figures 1 and 5. Although reaction is not limited exclusively to half of the sites, there is an order of magnitude difference in the rates of incorporation at neighboring catalytic centers. This is in contrast to the acylation of cysteine-149 of glyceraldehyde-phosphate dehydrogenase where only two of four centers are derivatized (Stallcup and Koshland, 1973). It is not possible at this stage to say whether any one of the four possible explanations earlier suggested for half-of-the-sites reactivity of glyceraldehyde-phosphate dehydrogenase applies to UDPGDH (Conway and Koshland, 1968). It is interesting that the introduction of a charged group such as carboxymethyl prohibits alkylation beyond that occurring at the catalytic site.

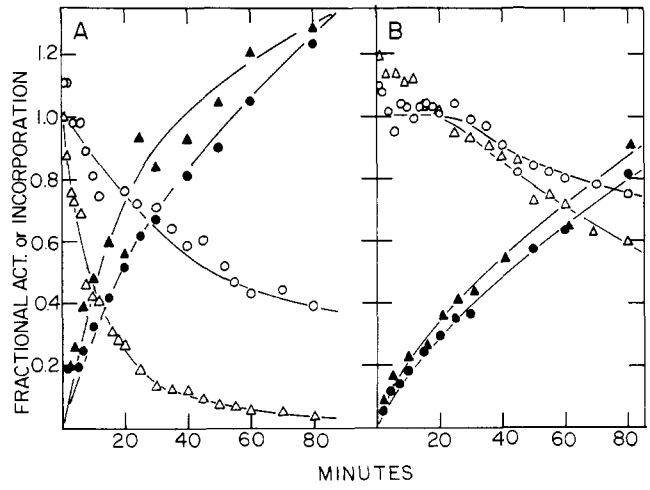


FIGURE 7: Effect of the combined presence of NADH and UDP, Figure 7A, or NADH and UDPX, Figure 7B, on carboxamidomethylation and activity loss. For Figure 7A, components were present at the following concentrations: enzyme, 10 μN; UDP, 3000 μM, (●, ○); or UDP, 3000 μM with NADH, 100 μM (▲, △). For Figure 7B, the same conditions prevailed except that UDPX at 100 μM replaced UDP.

One might infer from this that the noncatalytic site thiol groups are sufficiently close to those at the catalytic sites to experience direct electrostatic effects. From Figures 1 and 5, it is also apparent that alkylated catalytic center thiol groups influence the catalytic power of neighboring catalytic centers. Specifically, Figure 1 shows that loss of activity does not directly follow the alkylation of thiols as given by the uppermost decay line. The expression for the fractional activity loss, f, is given by eq 4

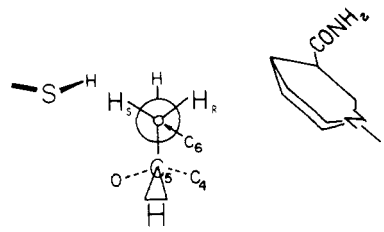
$$f = e^{-k_1 t} - \frac{k_1 \phi}{2(k_2 - k_1)}(e^{-k_1 t} - e^{-k_2 t}) \qquad (4)$$

where ϕ represents interaction between blocked and unblocked sites. This parameter was introduced by Stallcup and Koshland; in our formulation ϕ has twice the numerical value of the parameter as defined by Stallcup and Koshland (Stallcup and Koshland, 1972). A value of $\phi = 1$ indicates no influence of the alkylated site on the free site, while a value of $\phi = 0$ means that a free site whose neighbor is blocked is catalytically inactive. For the experiments summarized in Figure 1, it appears that carboxymethylation of one site caused a 40% reduction in the catalytic efficiency of its neighbor. On the other hand, from Figure 5 carboxamidomethylation appears to produce a 50 to 80% reduction in neighboring site catalytic activity. Close inspection of the actual inactivation decay shows that the data cannot be accurately described by an expression as simple as eq 4. The loss of activity at low incorporations is not as great as that predicted by eq 4, while at greater incorporations it is significantly greater. This may even be the case with carboxymethylation, though the effect is not as strong. It is possible to explain the excess inhibition of catalytic activity at higher levels of incorporation seen in Figure 5 either in terms of the interaction between pairs of subunits as well as within pairs, or in terms of a negative influence of alkylation at noncatalytic thiol groups. The latter possibility is unlikely since incorporation at noncatalytic thiols is negligible during the first 10 min of the reaction in the presence of NAD^+ or NADH, Figure 6, yet the activity loss far exceeds the incorporation. We have noticed that the degree of inhibition of catalytic power of a specified catalytic site caused by blocking a neighboring catalytic site is a variable quantity depending on the history

of the enzyme preparation. For example, the two preparations represented in Figure 5 show the same incorporation kinetics, but show significant differences in the inactivation kinetics. Both, however, are characterized by apparent ϕ values less than 1. Likewise, the inactivation kinetics for iodoacetamide inactivation in the presence of NAD^+ or NADH, Figure 6, require an apparent ϕ value of 0.2 or less.

As mentioned in the introductory section, the reaction of UDPGDH with DTNB also shows a burst of initial thiol blocking (Gainey et al., 1972). In this case the stoichiometry indicates that once two catalytic sites per hexamer react, the remaining four are slower to react. Our data show that three out of six sites are rapidly inactivated by alkylation, suggesting that the fundamental unit in the hexamer is a dimer, the hexamer being a trimer of such dimers. Preliminary equilibrium centrifugation work in this laboratory supports the notion that a dissociation process can be induced which yields dimers of the fundamental subunits in equilibrium with hexamers. These facts coupled with the present data and the knowledge that the substrates, UDPG and NAD, each bind with a stoichiometry of three per hexamer, support the classification of UDPGDH as a half-of-the-sites enzyme (Franzen et al., 1973). Some half-of-the-sites enzymes such as horse liver, alcohol dehydrogenase, and *E. coli* alkaline phosphatase exhibit the "burst" phenomenon in transient kinetics studies (Luisi and Favilla, 1972; Chappelet-Tordo et al., 1974). However, stopped-flow experiments on the enzyme-catalyzed oxidation of UDPG under a variety of mixing arrangements and concentration ratios of enzyme and substrates have given no indications of rapid initial transients.[2] Such negative rapid reaction kinetics results do not necessarily rule out a priming effect of one site on another, but may simply indicate that the steady-state hydride transfer steps have rate constants which are greater than that of the initial turnover.

The effects of enzyme substrates and inhibitors on the reactivity of the catalytic site thiols and other thiols is informative with respect to the topology of the catalytic center. It is significant that UDPG, UDPX, UDPGA, and UDP all serve to inhibit the reactivity of catalytic site thiols (data for UDPGA not shown) whereas NAD^+ and NADH either have no effect, as in the case of iodoacetate, or an activating effect, as with iodoacetamide. From the extensive work of Kirkwood and his collaborators on the mechanism of the two stage oxidation, it is clear that UDPG must be within covalent bond formation distance of the critical thiol group (Nelsestuen and Kirkwood, 1971; Ridley and Kirkwood, 1973; Ridley et al., 1975). Presumably the other ligands, UDPX, UDPGA, and UDP, also bind at the same site as UDPG, though there may be some question as to where UDPX most tightly binds (Chen et al., 1974; Gainey and Phelps, 1975). The data of this paper are consistent with the formulation of the scheme which positions



NAD^+ (NADH) at a location more distant than UDPG from the catalytic site thiol group. The involvement of Schiff base formation with a participating amino group from a nearby lysine would not alter the basic argument that carbon 6 of the glucose moiety or of the aldehyde formed from glucose is juxtaposed between the essential thiol and NAD^+ (Ordman and Kirkwood, 1976). The conformation pictured for the region of the glucose moiety of interest is that purported to be the free solution conformation for glucose in UDPG on the basis of NMR evidence (Sarma et al., 1973). Whether this conformation or some other characterizes the bound substrate does not necessarily upset the hypothesis offered here regarding the topological relationship of the substrates and the active thiol.

The activating effects of NAD^+ and NADH on the reaction of the catalytic site with iodoacetamide are striking, but the influence of NAD^+ on such alkylations is not without precedent. For example, the reaction of iodoacetate with glyceraldehyde phosphate dehydrogenase is enhanced by NAD^+, while that with iodoacetamide is inhibited (Mac Quarrie and Bernhard, 1971). Roles are reversed in the case of UDPGDH. Also, as mentioned in the introductory section, NAD^+ stimulates the early transient in the reaction of DTNB with the UDPGDH (Gainey et al., 1972). This action of nicotinamide coenzymes must have some significance regarding the catalytic process of the native enzyme, though at present it is unclear just what this significance is. The coenzymes also display longer range effects, reducing the reactivity of non-catalytic thiols to iodoacetamide. Thus, the conformational alterations produced by NAD^+ and NADH in the protein are pervasive.

The inhibition of the alkylation with iodoacetate by UDPG, UDPX, and UDP indicates that each of the ligands binds to the catalytic site, Figures 3 and 4, and unpublished data. It is striking that the protection of the enzymatic activity against iodoacetate is so strong. This is probably due to electrostatic repulsion between the negatively charged reagent and the negatively charged ligands. The inhibition of the alkylation with iodoacetamide by UDPG, UDPX, and UDP, Figures 6 and 7, shows that this uncharged reagent is less hindered in attacking the enzyme than is iodoacetate. Iodoacetamide more readily reacts at the catalytic site thiol groups most likely because it is not electrostatically repelled by the bound ligands. Moreover it can react with other thiols on the enzyme, and such reaction may weaken the binding potential for the protective ligand, thereby triggering reaction at the catalytic center. This inductive effect is especially prominent in the incorporation and inactivation curves for inhibition by UDPX, Figure 7B. Incorporation progresses from the onset of mixing, but enzyme activity only starts to drop after 0.3 to 0.4 carboxamidomethyl groups have been introduced per subunit. Whether UDPX binds most firmly to an allosteric site on the enzyme or to the catalytic site cannot yet be answered unequivocally. Arguments for UDPX acting as an enzyme inhibitor by direct competition with UDPG at the catalytic site and by indirect action propagated from an allosteric site have been promulgated elsewhere (Chen et al., 1974; Gainey and Phelps, 1975). The data of this paper can be interpreted either way.

The effects observed in the presence of the two enzyme inhibitors can be interpreted in terms of the conclusions reached earlier regarding the topology of the active center. From Figure 7A, we see that the activating effect that NADH has on alkylation of the catalytic site with iodoacetamide is partially blocked by UDP, which is considered to bind at the active site. Thus NADH can partially overcome the inhibition of UDP on the alkylation with iodoacetamide. On the other hand, UDPX, containing the xylose sugar moiety not possessed by UDP,

[2] These unpublished experiments were carried out by one of the authors (J.S.F.) in the laboratory of Professor P. L. Luisi at the E.T.H.-Zurich.

essentially completely masks the influence of bound NADH. These findings support the idea that the sugar moiety of a UDP-sugar intervenes spatially between the nicotinamide coenzyme and the catalytic site thiol.

In summary, UDPGDH bears many of the marks of the half-of-the-sites enzymes and may operate catalytically by a flip-flop mechanism as recently suggested (Ordman and Kirkwood, 1976). It is clear that the catalytic site thiol groups interact with each other when alkylated. Events at one site are perceived by another site, this being a basic ingredient of any flip-flop mechanisms.

Acknowledgment

The technical assistance of Marie Bowser is gratefully acknowledged for certain aspects of the research reported here.

References

Chappelet-Tordo, D., Iwatsubo, M., and Lazdunski, M. (1974), *Biochemistry 13,* 3754.

Chen, A., Marchetti, P., Weingarten, M., Franzen, J. S., and Feingold, D. S. (1974), *Carbohydr. Res. 37,* 155.

Conway, A., and Koshland, Jr., D. E. (1968), *Biochemistry 7,* 4011.

Franzen, J. S., Kuo, I., Eichler, A. J., and Feingold, D. S. (1973), *Biochem. Biophys. Res. Commun. 50,* 517.

Frost, A. A., and Pearson, R. G. (1953), Kinetics and Mechanism, New York, N.Y., Wiley, pp 153–154.

Gainey, P. A., Pestell, T. C., and Phelps, C. F. (1972), *Biochem. J. 129,* 821.

Gainey, P. A., and Phelps, C. F. (1974), *Biochem. J. 141,* 667.

Gainey, P. A., and Phelps, C. F. (1975), *Biochem. J. 145,* 129.

Huang, Y. H. J., Roy-Burman, P., and Visser, D. W. (1971), *Biochem. Pharmacol. 20,* 2447.

Luisi, P. L., and Favilla, R. (1972), *Biochemistry 11,* 2303.

Mac Quarrie, R. A., and Bernhard, S. A. (1971), *Biochemistry 10,* 2456.

Nelsestuen, G. L., and Kirkwood, S. (1971), *J. Biol. Chem. 246,* 3828.

Neufeld, E., and Hall, C. W. (1965), *Biochem. Biophys. Res. Commun. 19,* 456.

Ordman, A. O., and Kirkwood, S. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1751.

Ridley, W. P., Houchins, J. P., and Kirkwood, S. (1975), *J. Biol. Chem. 250,* 8761.

Ridley, W. P., and Kirkwood, S. (1973), *Biochem. Biophys. Res. Commun. 54,* 955.

Sarma, R. H., Lee, C.-H., Hruska, F. E., and Wood, D. J. (1973), *FEBS Lett. 36,* 157.

Stallcup, W. B., and Koshland, Jr., D. E. (1972), *Biochem. Biophys. Res. Commun. 49,* 1108.

Stallcup, W. B., and Koshland, Jr., D. E. (1973), *J. Mol. Biol. 80,* 41.

Uram, M., Bowser, A. M., Feingold, D. S., and Lamy, F. (1972), *An. Asoc. Quim. Argent. 60,* 135.

Zalitas, J., and Feingold, D. S. (1968), *Biochem. Biophys. Res. Commun. 31,* 693.

Zalitas, J., and Feingold, D. S. (1969), *Arch. Biochem. Biophys. 132,* 457.

Nuclear Magnetic Resonance Studies of D_2O–Substrate Exchange Reactions Catalyzed by Glutamic Pyruvic and Glutamic Oxaloacetic Transaminases†

U. M. Babu and R. B. Johnston*

ABSTRACT: Nuclear magnetic resonance studies in D_2O (>90%) with glutamic pyruvate transaminase (GTP) (2.6.1.2) demonstrate that this enzyme catalyzes the rapid exchange of both the α and β hydrogens of L-alanine, the exchange of only one α hydrogen of glycine, and the β hydrogens of pyruvate and fluoropyruvate. When the β hydrogens of L-alanine undergo the enzyme-catalyzed exchange, the product may have 1, 2, or 3 of β hydrogens exchanged. The exchange is stimulated by the addition of catalytic amounts of copartner of transaminations reaction. A mechanism is proposed for an extention of the conjugated system to include the α and β carbons to explain the labilization of the β hydrogens.

The most widely accepted mechanism of action for enzymatic transamination was proposed independently by Braunstein and Snell (Braunstein and Shemyakin, 1953; Metzler et al., 1954). This mechanism which was based prinicipally on extensive studies with model compounds involves the formation of the Schiff's base which results in a labilization of the α hydrogen of the amino acid. Early experiments by Grisolia and Burris (1954) and by Hilton et al. (1954) demonstrated that GOT[1] catalyzes the α-hydrogen exchange of glutamate. The first suggestion that the enzyme mechanism may be more complicated in that an interaction may also occur at the hydrogens of the β carbon was the observation of Oshima and Tamiya (1959, 1961) who found that the infrared spectra of L-alanine isolated after ion-exchange chromatography after it had been

† From the Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588. *Received August 9, 1974.*

[1] Abbreviations used: NMR, nuclear magnetic resonance; GPT and GOT, glutamic pyruvic and glutamic oxaloacetic transaminases.