Biochimica et Biophysica Acta, 614 (1980) 242—255

BBA 69056

INDUCED VERSUS PRE-EXISTING ASYMMETRY MODELS FOR THE HALF-OF-THE-SITES REACTIVITY EFFECT IN BOVINE LIVER URIDINE DIPHOSPHOGLUCOSE DEHYDROGENASE

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(Received December 10th, 1979)

Key words: Asymmetry model; Half-of-the-sites reactivity; Uridine diphosphoglucose dehydrogenase; (Bovine liver)

Summary

Half-of-the-sites reactivity of the catalytic site thiol groups of UDPglucose dehydrogenase (UDPglucose:NAD^+ 6-oxidoreductase, EC 1.1.1.22) can be ascribed either to the induction of conformational asymmetry following derivatization of one half of the subunits or to intrinsic conformational differences in the subunits of the native enzyme. If the half-sites reactivity behavior is due to induction effects, the magnitude of the induction could be expected to depend on the nature of the covalent modification. On the other hand, if the half-sites reactivity behavior is due to pre-existing asymmetry and there is no communication between catalytic centers, the properties of unmodified subunits should be independent of the nature of the covalent derivative introduced on the modified subunits. According to the induced asymmetry hypothesis, the catalytic activity of half-sites modified enzyme might be different for different covalent modifications, whereas for the rigid pre-existing asymmetry hypothesis the catalytic activity of half-sites modified enzyme should be the same regardless of the modifying group. During the course of catalytic site thiol group modification by a number of thiol specific reagents, the loss of enzyme activity was equivalent to the degree of modification for most of the reagents employed. However, with iodoacetate and 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid, half-sites modification of UDPglucose dehydrogenase

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Abbreviations: BUP, *p*-bromoacetamidophenyl uridyl pyrophosphate; DTDN, 6,6′-dithiodinicotinate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); IAEDANS, 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid; IAF, 5-iodoacetamidofluorescein; NEM, *N*-ethylmaleimide; NTCB, 2-nitro-5-thiocyanobenzoic acid.

reduced catalytic activity by 58 and 78%, respectively, of the initial activity. These observations are consistent with a model in which there is communication between catalytic sites. Electron microscopy shows that the six subunits of UDPglucose dehydrogenase are arranged as a hexagonal planar ensemble.

Introduction

Half-of-the-sites reactivity in bovine liver UDPglucose dehydrogenase (UDPglucose:NAD^+ 6-oxidoreductase, EC 1.1.1.22) has been demonstrated both with respect to substrate binding [1,2] and catalytic site thiol group reactivity [3,4]. The enzyme catalyzes the two-stage, four-electron-transfer oxidation of UDPglucose via an enzyme-bound aldehyde intermediate to UDPglucuronate [5]. The essential thiol at the catalytic center is involved in the formation of a hemithioacetal linkage with the aldehyde intermediate and is regenerated by hydrolytic cleavage of the thio ester linkage formed in the second stage. This enzyme is one of a growing list of proteins which display half-of-the-sites behavior, as documented for example in review articles by Lazdunski [6] and Levitski and Koshland [7].

Half-of-the-sites reactivity in proteins can be explained on the basis of a number of underlying mechanisms [8]. If it is assumed that the six subunits of UDPglucose dehydrogenase are identical in primary structure [9] and that the catalytic sites are distant from each other, the two most likely mechanisms for explaining half-of-the-sites behavior in UDPglucose dehydrogenase are the so-called pre-existing asymmetry and induced asymmetry models. In the former model, the oligomer consists of two conformationally different classes of subunits, one of which reacts with a modifying reagent more rapidly than the other. According to the simple version of this model, whatever happens at one subunit has no influence on events that might occur on neighboring subunits. In the latter model, induced asymmetry, the subunits are conformationally identical in the native enzyme, but modification of any subunit induces a conformational change in its neighbor(s) with a resultant alteration in reactivity or other properties of the neighbor. Some approaches for providing evidence on which to make a choice between these two mechanisms for any particular half-of-the-sites enzyme have been elaborated and applied, though not always with unanimity about the interpretation of results [10—14].

In this paper we present evidence to support the view that the half-of-the-sites reactivity observed with UDPglucose dehydrogenase involves the induction of a conformational change in the subunit adjacent to the one which undergoes catalytic site thiol modification. Any pre-existing asymmetry model in which the bonding domains between subunits are rigid and do not allow for events at one subunit to influence events at another is ruled out. On the basis of these conclusions and electron microscopy results, which show that the hexameric enzyme has a hexagonal planar arrangement, it is proposed that the hexamer has D_3 (or 32) rather than C_6 (or 6) point group symmetry.

Results

Materials. Iodo[^{3}H]acetate and iodo[2-^{14}C]acetate with respective specific radioactivities of 100 and 14.7 mCi/mmol were purchased from New England

Nuclear (Boston, MA) and were recrystallized from *n*-hexane prior to use. Iodo[2-^{14}C]acetamide (9.35 mCi/mmol) and *N*-[*ethyl*-1-^{14}C]ethyl maleimide (525 mCi/mmol), were also obtained from New England Nuclear. The former was recrystallized from chloroform. Fresh stock solutions of the latter were prepared directly just before use by diluting an aliquot of NEM as supplied in pentane solution with the appropriate buffer. The NEM concentration of the stock solution was determined by reacting a known volume with an excess of glutathione. The thiol content of glutathione in the solution was determined by reaction with DTNB before and after NEM addition, and the NEM concentration of the stock solution was calculated from the difference in color formation. DTNB and DTDN were supplied by Aldrich (Milwaukee, WI) and NTCB was prepared according to established procedures [15].

Nonradioactive IAEDANS and IAF were bought from Molecular Probes (Plano, TX). [^{3}H]IAEDANS was prepared using [^{3}H]iodoacetic acid as the radiolabeled starting component according to the method of Hudson and Weber [16]. [^{14}C]IAF was prepared by reacting fluorescein amine, isomer I, with chloroacetyl chloride followed by halogen exchange reaction with NaI. Chloro[1-^{14}C]acetyl chloride was prepared from chloro[1-^{14}C]acetic acid (2.8 mCi/mmol purchased from New England Nuclear (Boston, MA)), by refluxing 0.2 mmol free acid under anhydrous conditions with 1.5 equivalent of thionyl chloride for 2 h in 1.5 ml benzene containing catalytic amounts of dimethylformamide [17,18]. After cooling to 23°C, one equivalent of fluorescein amine suspended in 2 ml acetone and 2 equiv. sodium carbonate were added in succession. The mixture was stirred for 36 h at 23°C under anhydrous conditions. The solvent was removed, and the residue was dissolved in a 0.5 ml of dimethylformamide, transferred to 5 ml water and acidified with 6 N HCl. The resulting precipitate was washed with water and dried in vacuo. The product was purified on 100 μm preparative TLC plates (Analtech Silica Gel G from Fisher, Pittsburgh, PA) using benzene/ethyl acetate/acetic acid (5 : 5 : 1, v/v). The yellow highly fluorescent band of 5-chloroacetamidofluorescein, which ran ahead of the nonfluorescent free amine, was eluted with ethanol and the solvent was evaporated under reduced pressure. The resulting oil was dissolved in 0.5 ml dimethylformamide and precipitated as above. The dried precipitate was dissolved in 25 μl of dimethylformamide followed by the addition of 1.0 ml of dry acetone. A 3-fold excess of sodium iodide dissolved in 1.0 ml dry acetone was added. After stirring for 52 h the desired product, 5-iodoacetamidofluorescein, was precipitated with water. The precipitate, washed and dried as above, had absorbance, fluorescence and chromatographic properties identical to those of authentic 5-iodoacetamidofluorescien obtained commercially. The yield of product was usually about 30%.

BUP, both nonradioactive and labeled in the acetamido moiety with ^{14}C, was provided as a gift from Professor Perry Frey of Ohio State University.

The enzyme was prepared and assayed as described by Zalitas and Feingold [19] with modifications as indicated by Franzen et al. [3]. The reported experiments were carried out on a single preparation of the enzyme. Although different preparations of the enzyme vary slightly with respect to specific activity and rate of reaction with thiol modifying reagents, the general pattern of reactivity behavior is consistent with that reported here. This collection of

data, using various reagents and a single preparation of enzyme, allows one to be more confident about any observed differences in the response of the enzyme to different reagents. The enzyme preparation showed greater than 95% purity on polyacrylamide gel electrophoresis and had a specific activity of 3.5 units per mg, where 1 unit refers μmol UDPglucose oxidized per min. During the course of the investivation, the specific activity dropped to 3.0 units per mg. All enzyme concentrations were determined using a specific extinction coefficient of 0.98 ml · mg^{-1} · cm^{-1} and are reported as μN, i.e. the number of μmol of 52 000 dalton subunits per l.

Procedures. Incorporation with radiolabeled reagents was carried out as described earlier [3] with the following minor modifications. For the precipitation of enzyme, aliquots of the reaction mixture containing 25 μg of enzyme were added to 1 ml ice-chilled solution containing: 7 g Cl_3CCOOH/5 g phosphotungstic acid/50 ml 95% ethanol/50 ml water. The presence of ethanol did not influence the assay of the incorporation reaction, as checked by measurement of [^{14}C]carboxymethyl incorporation in the presence and in the absence of ethanol. However, when ethanol was omitted from the precipitation mixture, very high background values were obtained in the IAEDANS experiments. The ethanol apparently enhances the solubility of IAEDANS and permits complete removal of noncovalently bound reagent from the filters. Other alterations were made in the preparation of the sample-containing filters for scintillation counting. A small volume of water, 100 μl, was placed on each filter in the counting vials, and 0.5 ml 0.5 N quaternary ammonium hydroxide in toluene (Protosol, New England Nuclear) was added to each vial. The vials were allowed to stand for 60 min, after which 5 ml Biofluor scintillation fluid (New England Nuclear) was added, followed by 100 μl glacial acetic acid. The samples were then counted. The addition of glacial acetic acid quenched chemiluminesce effects and stabilized the homogeneous condition of the sample-containing scintillation cocktail. Without added acetic acid, occasional formation of flocculent precipitates in the counting vials yielded unreproducible results. Blank samples were prepared as described earlier [3]. The concentrations of reagent in the reagent solutions were determined gravimetrically for iodoacetate, and spectrophotometrically for IAEDANS, ϵ = 6100 $cm^{-1} \cdot M^{-1}$ at 337 nm [16]; IAF, ϵ = 85 000 $cm^{-1} \cdot M^{-1}$ [20] and BUP, ϵ = 16 000 $cm^{-1} \cdot M^{-1}$ [21]. As described above, NEM concentrations were determined by reaction with excess thiol and colorimetric quantitation of thiol loss. All incorporations are reported as mol of reagent covalently bound per mol of enzyme subunits.
units.

Reactions of the enzyme with NTCB were followed by monitoring the absorbance of solutions at 412 nm, ϵ = 14 100 $cm^{-1} \cdot M^{-1}$ [22]. The immediate product of the NTCB reaction is *S*-cyanylated enzyme. It is assumed that no loss of this initial product via elimination of nitrilo groups and formation of internal disulfide bonds occurred during the time course of the reaction. The reaction of the enzyme with DTDN is much faster than that with most of the other reagents employed in this study [4,9]. It was therefore not possible to sample the reaction mixture during the progress of the reaction with DTDN and assess accurately the level of remaining enzyme activity at the instant of

sampling. Since DTDN reacts quantitatively with the enzyme when the enzyme is in molar excess, various subequivalent amounts of DTDN were incubated with the enzyme for 2 h. Trial experiments showed that over this time course the reaction was complete, 2 mol of 5-carboxy-2-thiopyridone, $\epsilon = 10\,000$ $cm^{-1} \cdot M^{-1}$ at 344 nm [23], being released for each mol of DTDN present. Under the reaction conditions used with this reagent, incorporation is proportional to the number of internal disulfide bridges introduced per subunit [4]. The catalytic activity of each sample was determined at the end of the incubation period. Enzyme not treated with DTDN lost, at most, 5% of its original activity during the experiment.

Preparations of the enzyme for electron microscopy were negatively stained with 2% aqueous uranyl acetate. The specimen was prepared from enzymatically active UDPglucose dehydrogenase at a concentration of 10 μg/ml in 0.02 M sodium acetate, pH 5.5. The material was diluted 1 : 1 directly on the grid support membrane (Parlodion, Mallinckrodt Chemical Works, St. Louis, MO) with the staining solution. Specimens were examined in an AEI EM6B electron microscope at an accelerating voltage of 80 kV and were recorded at an instrument magnification as indicated in the legend to Fig. 3.

Results

The progress curves for the incorporation of alkyl groups and the consequent loss of enzyme activity are shown in Fig. 1 for the reaction of UPDglucose dehydrogenase with iodoacetate, IAEDANS and BUP. The carboxymethylation reaction described previously [3,4] was done in a different buffer and at a higher temperature than used in this study. Though the chemical reaction is the same for these three reagents, it is clear from Fig. 1 that the variation in alkylation rate is enormous. The very fast reaction with BUP is not unexpected. This compound is probable classifiable as an affinity reagent for UDPglucose dehydrogenase. As was observed for alkylation by iodoacetate, the alkylation with BUP is strongly inhibited by UDPxylose, moderately by UDPglucose, and slightly if at all by NAD^+ and NADH. The progress curves showing these inhibition results are not presented here. The reaction with BUP is restricted to catalytic centers, alkylation being limited to the introduction of one alkyl group per subunit accompanied by complete loss of enzyme activity. Inspection of Fig. 1 suggests the presence of a half-sites burst, a point which is made more convincing by comparison of the values of the rate constants enumerated in the legend of Fig 1, and by consideration of the analytical treatment of certain slopes of the progress curve as described further on. Like BUP, IAEDANS also reacts remarkably rapidly with the enzyme. This reaction, too, is inhibited by UDPxylose, is unaffected by NAD^+ and is strongly biphasic showing a clear half-sites burst. However, the IAEDANS reaction is not limited to the catalytic site thiols of the enzyme. If the reaction is carried out at length under the conditions of Fig. 1, or if a higher ratio of reagent to enzyme is used, the incorporation level goes well beyond one alkyl group per subunit. Furthermore, though at a total incorporation level of 0.5 one fluorescent tryptic peptide is found, when the total incorporation level is 1.0 other fluorescent peptides appear in minor amounts.

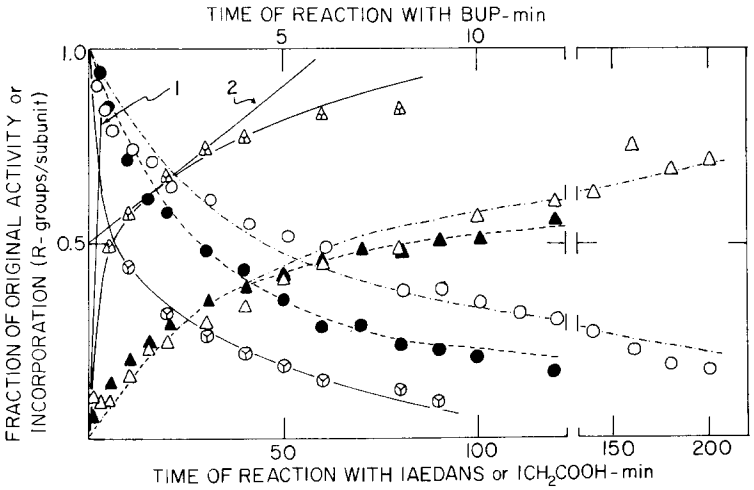

Fig. 1. Incorporation and inactivation progress curves for UDPglucose dehydrogenase reacted with BUP, trisected symbols, IAEDANS, filled symbols, and iodoacetate, open symbols. Reactions were performed at 25°C in 0.1 M potassium phosphate buffer, pH 8.0, with enzymes present at a concentration of 5.0 μN. Concentrations of reagents were 60 μM for BUP and IAEDANS, and 320 μM for iodoacetate. Triangles and circles represent incorporation and inactivation, respectively. The upper abscissa scale of 0—10 min applied to the BUP data, while the lower 0—200 min scale applies to the IAEDANS and iodoacetate data. The drawn incorporation and inactivation progress curves are constructed according to Eqns. 1 and 2 using the following kinetic parameter values: BUP (———), $k_1 = 600\ M^{-1} \cdot s^{-1}$, $k_2 = 60\ M^{-1} \cdot s^{-1}$, $\phi = 1.0$; IAEDANS (- - - - - -), $k_1 = 5.0\ M^{-1} \cdot s^{-1}$, $k_2 = 0.33\ M^{-1} \cdot s^{-1}$, $\phi = 0.45$; iodoacetate (●———●), $k_1 = 0.083\ M^{-1} \cdot s^{-1}$, $k_2 = 0.017\ M^{-1} \cdot s^{-1}$, $\phi = 0.75$. The slopes of the lines labeled 1 and 2 are used to evaluate an apparent biphasicity parameter.

The three alkylating reagents discussed above have different effects on the residual catalytic activity of the enzyme. For example, when the average incorporation is 0.5 alkyl group per subunit, the enzymatic activity has dropped to 50, 42%, and 25% of the initial value for reaction with BUP, iodoacetate and IAEDANS, respectively, see Fig. 1. The loss of activity observed with the incorporation of the IAEDANS group is due strictly to an effect on k_{cat} and not on K_m. Steady state kinetics measurements on native enzyme and enzyme having three covalent attached IAEDANS groups per hexamer yielded $K_m^{NAD^+}$ values of 220 and 200 μM, respectively, and $K_m^{UDPglucose}$ values of 15.6 and 15.0 μM, respectively.

The collective data on the reactivity and catalytic behavior of the enzyme treated with these reagents and the others included in this study are summarized in Table I. Comparison of the apparent second-order rate constants for the initial phase of the reaction, as listed in column 2, demonstrates the hyperreactivity of BUP, the reagent which is a substrate analog. The only other reagent of those we examined which reacts as rapidly as BUP is DTDN. For this reagent the reaction mechanism and product are distinct from those for other entries in the table. According to Gainey et al. [9], organomercurials also react very rapidly with UDPglucose dehydrogenase and appear to be nonspecific for catalytic site thiols. The catalytic site thiols also manifest high reactivity toward alkylating reagents having substantial hydrophobic character as is evidenced by the relatively large rate constants for IAF and IAEDANS in Table I.

TABLE I

SUMMARY OF INCORPORATION AND INACTIVATION PARAMETERS FOR THE REACTION OF THIOL SPECIFIC REAGENTS WITH UDP-GLUCOSE DEHYDROGENASE

Reagent	$k_1(M^{-1} \cdot s^{-1})$ *	Slope ratio ***	Remaining activity of half blocked enzyme (%)
BUP	600	27	50
DTDN	600 **	11 **	50
IAF	86	20	50
NTCB	17	24	50
NEM	10	14	50
IAEDANS	5.0	35	25
Iodoacetamide	0.69	7.5	50
Iodoacetate	0.20	26	42

* For BUP, IAEDANS, and iodoacetate the second-order-rate constants were determined by fitting the data of Fig. 1 to Eqn. 1. For all other entries, the rate constants were obtained by dividing the initial slope of the progress curves by the concentration of reagent.

** Entries taken from earlier work for reaction run at 20° C [4].

*** The quotient of the initial slope and that of the tangent drawn from the point 0, 0.5 (see text).

Though the progress curves are not all shown here, it is evident from examination of such curves that the reaction of UDPglucose dehydrogenase with the reagents listed in Table I is clearly biphasic, with one exception (iodoacetamide), and a burst of 0.5 group introduced per subunit is distinguishable. Since the reaction is not specific for catalytic site thiols with all of the reagents, it is not possible to evaluate a characteristic rate constant for the second phase of the reaction without assuming some kind of model. We choose therefore to introduce a completely empirical parameter as a measure of biphasicity. If one has a single phase first-order or pseudo-first-order process, it can be shown that the ratio of the initial rate (initial slope of the progress curve) to the slope of a line drawn tangent to the progress curve from 0.5 on the ordinate (expressed as fraction extent of reaction) has a constant value of 5.36, regardless of the value of the first-order rate constant (see Appendix). The two lines in question here are indicated as lines 1 and 2 in Fig. 1. The ratio of the slopes of these lines, S_1/S_2, would be 5.36 if the progress curve were that of a single first-order process. If the process were complex, involving a fast reaction accompanied or followed by slower reactions, the slope ratio would be greater than the above value. The experiments used to generate kinetic data have all been done under pseudo-first-order, or nearly pseudo-first-order conditions, initial concentrations of reagent being at least 12-fold greater than the enzyme subunit concentration. The analysis is therefore applicable to this system of reactions. The results are shown in column 3 of Table I (slope ratio). The numbers in column 3 indicate that all of the reagents, with the possible exception of iodoacetamide, react in a biphasic manner with the enzyme, and the slope ratios allow one to rank the reagents with respect to the degree of biphasicity they engender. It should be pointed out that if a reagent attacks the catalytic site thiols in a biphasic manner and attacks other thiols concomitantly, the observed slope ratio would be lower than if that reaction were limited exclusively to the catalytic site thiols. This caveat is applicable to all Table I apparent biphasicity parameter

values except those for the catalytic site specific reagents, iodoacetate and BUP. In view of the point just made, it is likely that the reaction with iodoacetamide is appreciably biphasic at the catalytic site thiols, even though the slope ratio value is only 7.5, since iodoacetamide is known to react at various locations on the enzyme [3].

The magnitude of the loss of catalytic activity produced by introducing thiol blocking groups at some but not all of the catalytic centers is listed in column 4 of Table I and is shown more fully in Fig. 2. For most of the reagents an average incorporation of 0.5 is associated with an equivalent loss of catalytic power, Fig. 2B. The notable exceptions are iodoacetate and IAEDANS, Fig. 2A. These exceptional cases provide the pivotal evidence for concluding that chemical events at a site on one subunit can induce effects at a like site on a neighboring subunit, as will be elaborated in the Discussion section. In the examples from Fig. 2B, the activity values at levels of incorporation greater than 0.5 lie above the line of slope equal to −1. This feature probably indicates that noncatalytic site thiols are derivatized significantly by these reagents at levels of incorporation greater than 0.5.

The results of our electron microscopic examination of UDPglucose dehydrogenase are seen in Fig. 3. This micrograph demonstrates that the hexameric enzyme has a quaternary structure which utilizes only the minimal number of intersubunit contacts possible for a closed symmetrical array of six identical asymmetric units. There is no evidence from the micrographs we have examined for hexamers having the potentially more stable trigonal prism or octahedral quaternary structures. From the original electron micrograph reproduced in the top part of Fig. 3, one can calculate an average subunit diameter of 3—4 nm. In practice the distances across a number of the hexamer arrays in the field was measured directly from the micrograph. One-third of the average value of these distances, divided by the appropriate magnification factor was used as the subunit diameter. Similar measurement of the polio virus particles in the same field yielded a diameter of 28 nm, in agreement with accepted measurements.

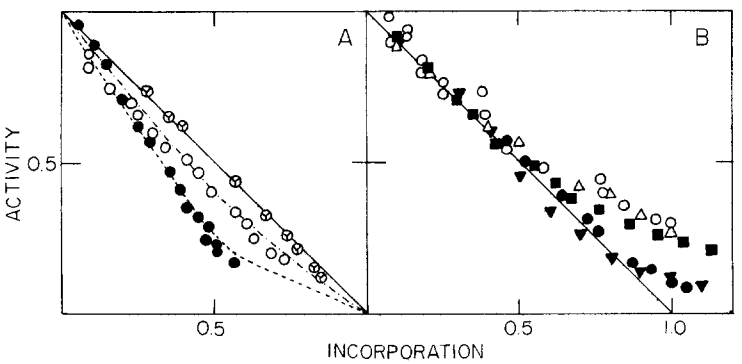

Fig. 2. Inactivation as a function of incorporation. The data of Fig. 1 are replotted in Fig. 2A. The trisected, open, and closed symbols represent BUP, iodoacetate, and IAEDANS data, respectively. The lines are drawn according to Eqns. 1 and 2 using the parameters indicated in the legend of Fig. 1. Fig. 2B shows the incorporation-inactivation data collected with DTDN, △; iodoacetate, ○; IAF, ●; NEM, ▼; and NTCB, ■.

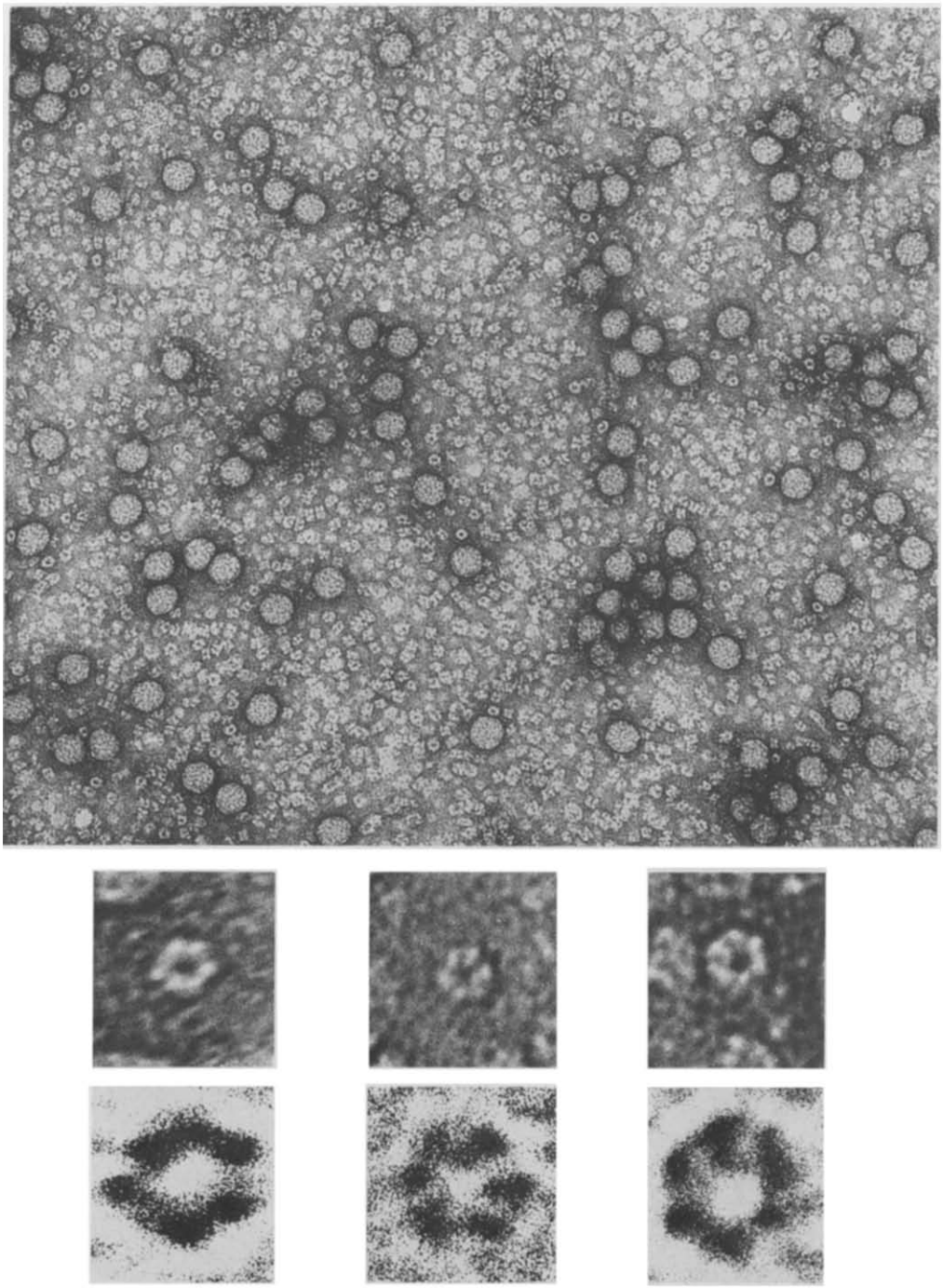

Fig. 3. Electron micrograph of UDPglucose dehydrogenase. The enzyme preparation was mixed with purified poliomyelitis virus particles (diameter 28 nm) and stained. Top photograph: instrument magnification 76530×, photographic enlargment 2×. Middle row: individual enzyme molecules from various fields; photographic magnification of original micrograph 10.5×. Bottom row: Kodalith (Eastman Kodak Co., Rochester, NY) inter negative prints of the enyzme molecules in the row above; additional photographic magnification of the corresponding middle row photograph, 2.5×.

Discussion

To distinguish between induced asymmetry and pre-existing asymmetry as underlying causes of apparent half-of-the-sites reactivity in proteins, Stallcup and Koshland [11] invented the 'reordered alkylation' experiment. They applied this approach to yeast glyceraldehyde-3-phosphate dehydrogenase to show that induction of effects across intersubunit bonding domains was involved in the operation of the half-sites effect for that enzyme.

We attempted to carry out the reordered alkylation experiment on UDP-glucose dehydrogenase using DTDN to make half-sites oxidized, reversibly blocked enzyme, followed by treatment with iodoacetate to block the remaining slowly reacting catalytic site thiols irreversibly. The scheme of the experiment requires that the second reaction, irreversible alkylation, be specific for the catalytic site thiol. Since carboxymethylation of half-sites oxidized enzyme was nonspecific, the reordered alkylation experiment could not be performed with iodoacetate [4]. Even when half-sites oxidation was followed by alkylation with BUP, the latter reaction occurred at thiols in addition to the one at the catalytic site. For example, alkylation of half-sites oxidized enzyme with BUP, although slower than that of native enzyme, led to an incorporation greater than the expected limit of 0.5 and a significant amount of enzyme activity remained.

Although we have not yet been able to do the reordered alkylation experiment with UDPglucose dehydrogenase, as will be shown in the following discussion, we have been able to show that alkylation of the catalytic site thiol groups of one subunit influences distant catalytic sites on neighboring subunits. For the sake of simplicity we develop our reasoning in terms of a dimeric enzyme, with the view that the hexameric UDPglucose dehydrogenase is best conceived as a trimer of such dimers. Support for this view is presented later. The monomers which comprise the native dimeric enzyme can be considered to be conformationally identical (pre-existing symmetry) or different (pre-existing asymmetry). In the case of pre-existing symmetry, modification of any catalytic site thiols would be characterized by an intrinsic second-order rate constant k_1, and modification at a catalytic site thiol on a subunit paired to an already modified subunit would be characterized by an intrinsic second-order rate constant k_2. Half-of-the-sites reactivity would result if k_2 were less than k_1. The kinetics for the modification reaction according to this model are described by Eqn. 1, where R represents the number of alkyl groups introduced per subunit, and A_0 is the concentration of modifying reagents [3].

$$R = 1 - e^{-2k_1A_0t} - \frac{k_1}{k_2 - 2k_1}(e^{-2k_1A_0t} - e^{-k_2A_0t}) \quad (1)$$

It would not be surprising to find that the induction that caused the change in the reaction rate constant also influenced k_{cat} for the catalytic site of the unalkylated pair member. The magnitude of this influence is expressed by the introduction of an inactivation-incorporation parameter, ϕ, which a priori could vary from 1.0 to zero (apparent inhibition), or could have values greater than 1.0 (apparent activation). Since ϕ represents an inductive effect, its magnitude could be expected to vary with the nature of the inducer, the thiol

blocking group. The kinetics of inactivation are given by Eqn. 2, where f represents the fraction of activity remaining.

$$f = e^{-2k_1A_0t} + \frac{\phi k_1}{k_2 - 2k_1}(e^{-2k_1A_0t} - e^{-k_2A_0t}) \quad (2)$$

If our model enzyme dimer is composed of two conformationally different subunits, a distinct second-order rate constant for modification could be assigned to each subunit and, as well, two specific catalytic constants, ρk_{cat} and σk_{cat}, could be assigned, where k_{cat} is the average catalytic constant for the subunits and $\rho + \sigma = 2$. We associate ρk_{cat} with the rapidly alkylated, k_1 subunits and σk_{cat} with the slowly alkylated, k_2 subunits. For this case the incorporation and inactivation kinetics are described by Eqns. 3 and 4 [3].

$$R = 1 - \tfrac{1}{2}(e^{-k_1A_0t} + e^{-k_2A_0t}) \quad (3)$$

$$f = e^{-k_1A_0t} - \frac{\sigma}{2}(e^{-k_1A_0t} - e^{-k_2A_0t}) \quad (4)$$

If it is further asserted that for the pre-existing asymmetry model, alkylation at one subunit does not communicate any effect to the other subunit, then at a given level of incorporation the remaining activity, though dependent on the value of the inactivation-incorporation parameter, σ, will be independent of the nature of blocking group on the thiol. That is to say, inactivation will be characterized by the same value of σ for all reagents.

The diagnosis for induction, or communication between subunits, therefore is to determine whether ϕ (or σ) values evaluated for a variety of thiol blocking reagents are all the same or different. If ϕ (or σ) is invariant, one cannot draw any conclusion. However, if the inactivation-incorporation parameter depends on the nature of the blocking group, one can conclude that subunits respond to what occurs on their partner subunits. Though it is possible in this latter situation to affirm that an induction process occurred, it is still not possible to decide whether the monomers of the dimer were originally conformationally identical or non-identical.

The Results section shows that alkylation by the affinity reagent BUP occurs with distinctly different values of k_1 and k_2, $S_1/S_2 = 27$. Since in Fig. 2A the inactivation-incorporation data for this reagent falls on the line joining the coordinates 0.1 1.0, ϕ (or σ) must equal 1.0. For a number of other reagents, iodoacetamide, IAF, NEM, NTCB and DTDN, ϕ is also equal to 1.0 as judged from the low incorporation portions of the inactivation-incorporation profiles of Fig. 2B. These latter reagents are only relatively specific since at higher incorporations the corresponding data points of Fig. 2 fall above the $\phi = 1$ line. In an earlier study we reported a ϕ value significantly less than 1.0 for iodoacetamide [3]. The presently reported experiments with iodoacetamide have been carried out under conditions wherein the inactivation rate was lower than in the earlier study, thus reducing the possibility that inactivation proceeded significantly from the time of sample withdrawal to the time of initiation of enzyme assay. In view of the particular care taken to obtain precise inactivation curves in this present study, the current data should probably be considered more reliable for application to the question addressed in this paper.

The inactivation-incorporation profiles for iodoacetate, a reagent specific for catalytic site thiols, and IAEDANS, a reagent relatively specific for catalytic site thiols, are distinct from those for the other reagents. For iodoacetate, a best fit to Eqns. 1 and 2 for the inactivation and incorporation data of Fig. 1 is obtained with $k_1 = 0.083\ M^{-1} \cdot s^{-1}$, $k_2 = 0.017\ M^{-1} \cdot s^{-1}$, and $\phi = 0.75$. For IAEDANS, assuming virtually complete specificity over the period of time covered by the reaction shown in Fig. 1, the corresponding parameter values are $k_1 = 5.0\ M^{-1} \cdot s^{-1}$, $k_2 = 0.33\ M^{-1} \cdot s^{-1}$, and $\phi = 0.45$. These data shown that ϕ is reagent-variable (and therefore can properly be identified as ϕ and not as σ). Hence, there is communication between catalytic centers in UDPglucose dehydrogenase. These data consequently support the induced asymmetry model for half-of-the-sites reactivity and allow us to rule out a rigid pre-existing asymmetry model. They do not, however, disallow the possibility of a pre-existing asymmetry with communication between sites.

It is of interest that the many blocking groups which have the same interaction parameter value, $\phi = 1.0$, are very different chemically: charged, uncharged, large, small, polar, hydrophobic, aromatic, non aromatic. There is nothing evidently common about the nature of the IAEDANS and carboxymethyl groups ($\phi \neq 1$) vis a vis the nature of the other groups ($\phi = 1$) that leads one to make any predictions about the detailed nature of the catalytic site and the kinds of modifications in the environs of a catalytic site which would induce a change in neighboring subunits. Hence, we can offer no satisfactory explanation for why some groups are inductive, $\phi \neq 1$, and some (most of those we have studied) are not, $\phi = 1.0$.

In the previous discussion we have treated the covalent modification reactions as second-order processes. However, if each of the mofidication steps proceeded through the formation of a stable enzyme-reagent intermediate complex, the rate constant k_1 and k_2 would be composed of a set of constants and would be dependent on the concentration of reagent. However, if the reagent concentration remained virtually unchanged (pseudo-first-order conditions), the collection of constants could be treated as a second-order rate constant and the formalism of Eqns. 1—4 and the arguments developed with them would be maintained.

The inductive effects, suggested by the biphasisity of the incorporation kinetics and established by the variability in ϕ, must have a structural basis. Although a full understanding of these effects requires a detailed knownledge of quaternary and tertiary structure, preliminary concepts would be assisted by an approximate knowledge of the quaternary structure. Electron microscopic investigation has been fruitful in this regard, establishing that the hexameric enzyme is a hexagonal ensemble rather than a trigonal prism or an octahedron.

The hexagonal arrangement of subunits revealed at the resolution of the electron microscope, see Fig. 3, can be accommodated by either C_6 (6) or D_3 (32) point group symmetry. In the former symmetry group all the intersubunit domains must be identical and heterologous [24]. Even though the six bonding domains are identical for the C_6 symmetry ensemble, since each subunit is an asymmetric unit, any inductive effect resulting from covalently blocking the catalytic site thiol of one of the six subunits would not likely be propagated equally towards its two nearest neighbors. The second blocking

reaction would then have a low probability, low rate constant, for occurring on the inductively altered neighbor of the initially blocked subunit. However, the second blocking reaction would have a high probability, high rate constant, of occurring on any one of the other four empty subunits. If it occurred on the unaltered neighbor of the initially blocked subunit, three of the remaining four empty subunits would still have highly reactive catalytic site thiols. In turn third, fourth and fifth high-rate blocking reactions could occur on some population of the enzyme molecules. Consequently, although the incorporation kinetics would not be monophasic, the burst size, if observable would be greater than 0.5, i.e. greater than half-sites reactivity could be expected. On the other hand, if the hexagonal ensemble has D_3 point group symmetry, and induction takes place along one of the two kinds of isologous intersubunit bonding domains, a clear half-sites response is to be expected. This line of thinking about the half-sites behavior of UDPglucose dehydrogenase favors the assignment of D_3 symmetry to the enzyme hexamer. However, additional experimental evidence, as is obtainable from dissociation, hybridization, or crystallographic studies is necessary to establish that such is the case. If UDP-glucose dehydrogenase has hexagonal planar D_3 symmetry it is reasonable to describe the hexamer as a trimer of dimers to which the incorporation and inactivation kinetics equations presented above are applicable to a first approximation. It is conceivable that weaker interdimer interactions as well as the stronger intradimer interactions might also occur. The observation of such effects, if they are present, would require high precision measurement of the incorporation and inactivation progress curves.

Appendix

Demonstration that the biphasicity parameter defined in the text has a value of 5.36 for a wholly first-order process.

The fractional completion, R, of a first-order reaction is given by Eqn. A1. The initial slope, S_1, of the progress curve which Eqn. A1 describes is equal to the rate constant k.

$$R = 1 - e^{-kt} \tag{A1}$$

The tangent line, line 2 of Fig. 1, is described by Eqn. A2, where S_2 can be identified as the rate of the reaction at the time of tangency, $t = t_T$,

$$R_T = 0.5 + S_2 t \tag{A2}$$

The rate S_2 is also expressible as $k\, e^{-kt_T}$. Therefore we can write

$$S_2 = S_1\, e^{-S_1 t_T} \tag{A3}$$

At the point of tangency, Eqns. A1 and A2 can be combined to Eqn. A4. After solving Eqn. A3 for t_T, we can introduce the expression for it into Eqn. A4 to yield Eqn. A5

$$1 - e^{-S_1 t_T} = 0.5 + S_2 t_T \tag{A4}$$

$$\frac{S_1}{S_2} = e^{0.5(S_1/S_2) - 1} \tag{A5}$$

Eqn. A5 is an equality only when $S_1/S_2 = 5.36$.

Acknowledgments

The authors wish to thank Dr. Bruce A. Phillips for the generous gift of purified poliomyelitis virus particles. This work was supported by grants from the National Science Foundation (PCM 7708645) and the National Institutes of Health (AM 15332).

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