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Mechanism of Pigeon Liver Malic Enzyme. Reactivity of Class II Sulfhydryl Groups as a Conformational Probe for the "Half-of-the-Sites" Reactivity of the Enzyme with Bromopyruvate†

Terry A. Pry and Robert Y. Hsu*

ABSTRACT: A method is described for the selective masking of nonessential SH groups of pigeon liver malic enzyme by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) in the presence of $NADP^+$, Mn^{2+}, and the substrate analogue tartronate. The resulting enzyme derivative containing four intact class II SH groups per tetramer is fully active in the oxidative decarboxylation of malate. Alkylation of two class II SH groups by the affinity label bromopyruvate inactivates this enzyme and abolishes the reactivity of the two remaining groups toward this reagent, confirming the "half-of-the-sites" behavior reported in a previous communication (Chang, G. G., & Hsu, R. Y. (1977) *Biochemistry 16,* 311–320). In contrast, "all-of-the-sites" reactivity is obtained for DTNB, NEM, iodoacetate, and iodoacetamide, which cause inactivation by reacting with all of the class II SH groups. The reaction of the enzyme derivative with DTNB or NEM follows a pseudo-first-order process, yielding second-order rate constants of 0.49 and 0.13 mM^{-1} min^{-1}, respectively. The rate constant of DTNB is unaffected by partial modification of the enzyme with other "all-of-the-sites" reagents, whereas the rate constants of both reagents with the enzyme which has been exhaustively alkylated by bromopyruvate are decreased by 2.4-fold for DTNB and 3.6-fold for NEM. The reaction of partially alkylated malic enzyme containing fewer than two bound pyruvyl residues per tetramer exhibited biphasic behavior, which can be accounted for by two parallel pseudo-first-order processes with rate constants corresponding to those of the unalkylated and dialkylated enzyme. The "half-of-the-sites" effect of bromopyruvate is interpreted on the basis of negative cooperativity resulting from specific conformation changes induced by the alkylating ligand.

Pigeon liver malic enzyme (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) is a tetramer composed of identical or nearly identical subunits (Nevaldine et al., 1974). In previous studies we have shown that this enzyme contains four independent and equivalent nucleotide binding sites (Hsu & Lardy, 1967a). The substrates $NADP^+$ and malate exhibit typical Michaelis–Menten (i.e., noncooperative) kinetic behavior in the oxidative decarboxylase reaction at a constant level of metal activator (Hsu et al., 1967). The initial velocity and product inhibition patterns are consistent with a sequential, ordered kinetic mechanism with $NADP^+$ adding first, followed by malate, and the release of CO_2, pyruvate, and NADPH as products (Hsu et al., 1967). More recently, the possibility of anticooperativity or nonidentical active sites is suggested by the following observations: (a) metal binding studies indicating the presence of two tight and two to four weak Mn^{2+} sites per enzyme tetramer (Hsu et al., 1976); (b) the apparent kinetic negative cooperativity of Mn^{2+}, potentiated by the substrate malate (Hsu et al., 1976); (c) a transient burst of enzyme-bound NADPH which equals approximately half of the active-site concentration (Reynolds et al., 1978); and (d) the "half-of-the-sites" reactivity of bromopyruvate, which inactivates the enzyme after alkylating two of the four "essential" SH groups (Chang & Hsu, 1977).

† From the Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York 13210. *Received March 31, 1978.* This research was supported in part by grants from the National Institutes of Health (AM-13390 and 5501 RR 054 0214).

Previously, the presence of a large number of SH groups on malic enzyme precluded a detailed analysis of the reaction of "essential" SH groups. This difficulty is overcome in the current study by using malic enzyme premodified with nonspecific reagents in the presence of tartronate to mask the "nonessential" SH groups without destroying enzymatic activity. The four "essential" SH groups of the premodified enzyme react independently with nonspecific reagents 5,5′-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, iodoacetate, and iodoacetamide, showing "all-of-the-sites" behavior. The two remaining SH groups on the bromopyruvate-inactivated, premodified enzyme react with both 5,5′-dithiobis(2-nitrobenzoic acid) and *N*-ethylmaleimide, but at rates significantly lower than the same groups on the active premodified enzyme. These results are interpreted in terms of the ligand-induced negative cooperativity of Koshland et al. (1966).

Materials and Methods

Materials. Pigeon liver malic enzyme was purified according to Hsu & Lardy (1967b). Preparations which exhibited homogeneity in the analytical ultracentrifuge were dialyzed exhaustively against 50 mM Tris-Cl (pH 7.0) containing 10% glycerol and 10 mM DTT[1] and stored at −20 °C for future use. These preparations have specific activities of 38 to 41 units/mg in the oxidative decarboxylation reaction. A molecular weight of 260 000 was used in all calculations (Nevaldine et al., 1974).

Sephadex G-25 (Pharmacia), DTNB, L-malic acid, triethanolamine-HCl (A grade), EDTA, IAA, tartronate, IAM, Tris (Calbiochem), NEM (Sigma), DTT, $NADP^+$ (P-L Biochemicals, Inc.), and *N*-ethyl[1-^{14}C]maleimide (New England Nuclear) were purchased from the above sources. Distilled, deionized water was used throughout this work. All reagent solutions were prepared fresh daily and brought to neutrality before use.

Methods. The oxidative decarboxylase activity was assayed according to Hsu & Lardy (1967b) at 30 °C. Protein concentration was determined at 278 nm (Hsu & Lardy, 1967b). The same extinction coefficient (0.86 for a 0.1% solution) was used for the native and derivatized enzymes, since this value was shown to be unaffected by modification of the enzyme with bromopyruvate, DTNB, or NEM in the control experiments. The concentration of DTNB was estimated by titration against excess DTT (Jocelyn, 1972). The concentration of NEM was monitored optically using an extinction coefficient of 620 at 305 nm (Jocelyn, 1972, p 142). Incorporation of ^{14}C-labeled NEM was calculated from the protein concentration and the radioactivity determined in a Beckman LS-150 liquid scintillation counter using 15 mL of Aquasol (New England Nuclear).

Purified malic enzyme was thawed and dialyzed against buffer A to remove DTT before each use. The premodified enzyme, which was used in all experiments, was prepared by incubating malic enzyme for 20 min at 24 °C in the presence of $MnCl_2$ (3.6 mM) $NADP^+$ (0.2 mM), and tartronate (9.1 mM). DTNB (100-fold molar excess) or NEM (0.5 mM) was then added and the incubation was continued at 24 °C for an additional 60 min. Under these conditions, the "nonessential" SH groups were derivatized but the "essential" SH groups were protected by the substrate analogue. Control studies showed that the unprotected enzyme underwent complete loss of activity. Unreacted DTNB was removed by passing the enzyme solution (0.8 mL) through a Sephadex G-25 column (1 × 20 cm) that had been previously equilibrated at 4 °C with the above preincubation medium without DTNB, whereas the reaction with NEM was quenched with DTT (10 mM). The premodified enzymes were then dialyzed overnight in buffer A at 4 °C to remove the protecting agents.

All linear plots shown in this paper were obtained by linear regression analysis.

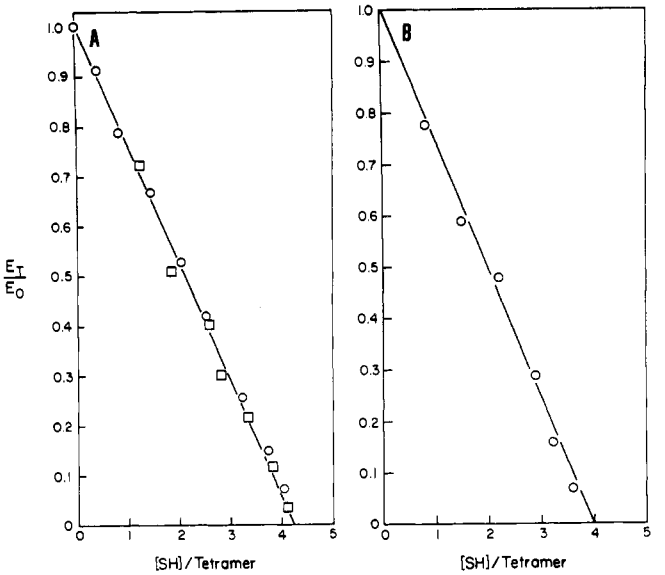

FIGURE 1: Correlation of fractional residual activity (E_T/E_0) and reaction of SH groups. The reactions were carried out by incubating premodified malic enzyme in buffer A with DTNB or [^{14}C]NEM at 24 °C. (A) Reaction of E_1 with DTNB. The different symbols represent results of separate experiments. (○) E_1 (specific activity, 41.9), 11.54 μM, DTNB, 0.34 mM; (□) E_1 (specific activity, 39.5), 11.33 μM, DTNB, 0.34 mM. Oxidative decarboxylase activity was determined on samples withdrawn at time intervals. The number of SH groups reacted per tetramer was determined by monitoring the release of thionitrobenzoic acid at 412 nm using an extinction coefficient of 13 600 M^{-1} cm^{-1} (Tang & Hsu, 1974). (B) Reaction of E_2 with [^{14}C]NEM. E_2 (specific activity, 38), 37.2 μM; [^{14}C]NEM, 0.5 mM (7.32 × 10^5 cpm/μmol). Samples were taken during the incubation, quenched with DTT (20 mM), diluted to 1.0 mL with cold buffer A, and dialyzed against the same buffer overnight to remove unbound radioactivity. The dialyzed samples were used for measurements of oxidative decarboxylase activity and radioisotope incorporation as described in Methods.

Results

Malic enzyme used in these studies was premodified by incubating with unlabeled DTNB or NEM in the presence of protecting agents tartronate, $NADP^+$, and Mn^{2+} by a procedure described in Methods. This technique provided an effective means of masking the "nonessential" SH groups, yielding E_1 (DTNB-premodified) and E_2 (NEM-premodified) which were fully active in the oxidative decarboxylation reaction. Typical titration behaviors of the premodified enzymes with excess DTNB and NEM are shown in Figure 1. In both cases, the loss of oxidative decarboxylase activity was proportional to the extent of reagent incorporation, and complete inactivation accompanied the modification of 4.2 SH groups per E_1 tetramer and 3.96 SH groups per E_2 tetramer. The inactivated enzyme was unreactive toward a second aliquot of the reagent, indicating the absence of additional titratable SH groups. These results agree well with the presence of four class II SH groups per tetramer, previously shown in our laboratory (Tang & Hsu, 1974). Moreover, these reagents reacted independently and equivalently in an "all-of-the-sites"[2] manner,

[1] Abbreviations used: buffer A, 50 mM Tris-Cl, pH 7.0; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; IAM, iodoacetamide; DTT, dithiothreitol.

[2] The nomenclature was that of Levitski & Koshland (1976).

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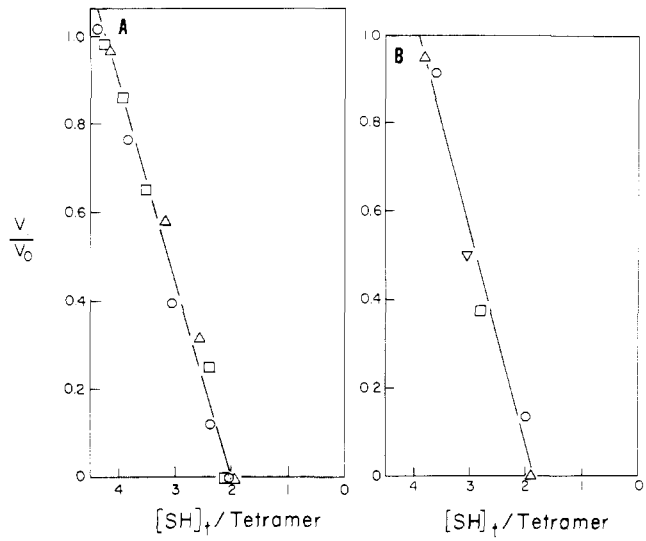

FIGURE 2: The loss of SH groups as a function of previous inactivation by bromopyruvate. The inactivated enzymes were prepared by incubating E_1 and E_2 (7–37 μM) in buffer A at 24 °C with 1.0 and 5.3 mM bromopyruvate, respectively. Samples were taken at time intervals, chilled on ice, and dialyzed at 4 °C against buffer A overnight. The reaction of E_2 was quenched by 10 mM DTT before chilling. The E_1 and E_2 samples at different stages of inactivation were assayed for oxidative decarboxylase activity, then titrated with excess DTNB (0.345 mM), or 0.5 mM [^{14}C]NEM (0.26 μmol, 7–11 × 10^5 cpm/μmol), respectively, at 24 °C. The DTNB reaction was monitored at 412 nm and the [^{14}C]NEM reaction was quantitated by aliquoting at time intervals, quenched with 10 mM DTT, dialyzed, and analyzed for radioisotope incorporation as shown in Methods. Each experimental point represents the total titratable SH groups on an enzyme sample, $[SH]_t$, which was previously inactivated by bromopyruvate to give the indicated value of V_i/V_0, the ratio of specific activities of the inactivated and fully activated enzymes. For the purpose of this calculation, the specific activity of the fully active enzyme was normalized to a value of 40 units/mg of protein. The control samples ($V_i/V_0 \simeq 1$) were titrated without bromopyruvate treatment (DTNB), or with 10 mM DTT added before bromopyruvate treatment (NEM). The different symbols represent results of separate experiments: (A) Titration with DTNB; (B) titration with NEM.

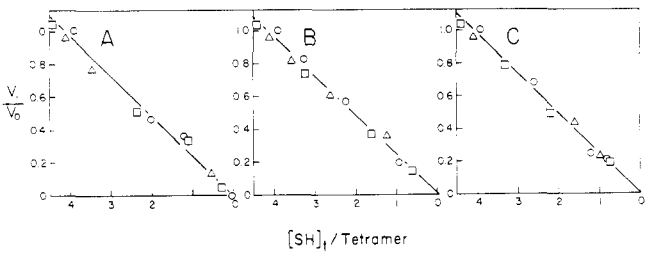

FIGURE 3: The loss of SH groups as a function of previous inactivation by iodoacetate, iodoacetamide, and *N*-ethylmaleimide. The incubations were carried out with E_1 as described in Figure 2A, except that bromopyruvate was replaced by iodoacetate (12 mM, A), iodoacetamide (12 mM, B), or NEM (0.8 mM, C); DTNB was used as the titrant.

in contrast to the "half-of-the-sites" behavior of the affinity label bromopyruvate (Chang & Hsu, 1977).

In a preliminary experiment, E_1 (15.3 μM) was incubated with 1.2 mM bromopyruvate in buffer A at 24 °C. Samples were withdrawn following complete inactivation (approximately 80 min) and after an additional 260 min, and dialyzed against buffer A to remove unbound bromopyruvate. The enzyme incubated for the shorter duration yielded 2.05 DTNB-sensitive SH groups per tetramer which was not reduced by incubation with bromopyruvate for an additional 260 min. This result indicates that the unalkylated SH groups of the bromopyruvate-inactivated enzyme are no longer susceptible to the halo acid, but are still reactive toward DTNB.

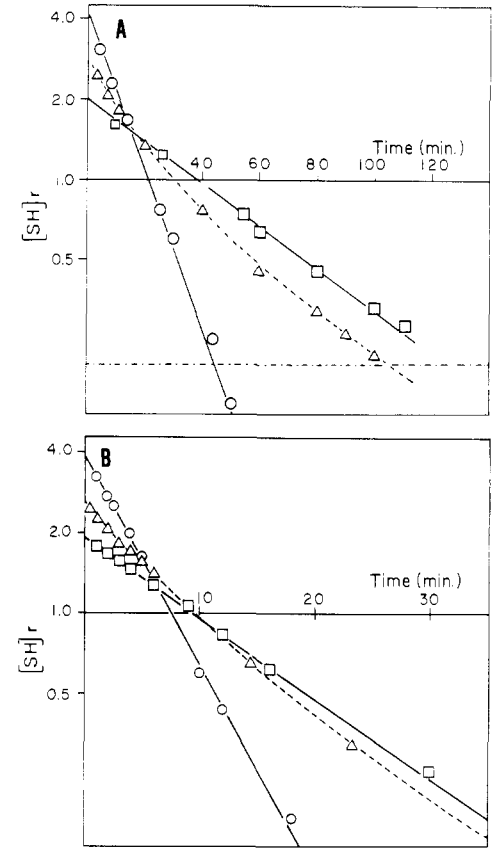

FIGURE 4: Pseudo-first-order plots of the reaction of bromopyruvate-inactivated malic enzyme with DTNB and [^{14}C]NEM. The kinetic data were taken from the progress curves of titration experiments described in Figure 2. $[SH]_r$, the number of SH groups remaining per enzyme tetramer, was obtained as the difference between the SH groups initially present on each sample, $[SH]_t$, and the amount reacted at time t. (A) [^{14}C]NEM titration: (○) active E_1 control ($V_i/V_0 = 1.0$); (□) E_2 completely inactivated by bromopyruvate ($V_i/V_0 = 0$); and (△) E_2 partially inactivated by bromopyruvate ($V_i/V_0 = 0.37$). (B) DTNB titration: (○) active E_1 control ($V_i/V_0 = 1.0$); (□) E_1 completely inactivated by bromopyruvate ($V_i/V_0 = 0$); and (△) E_1 partially inactivated by bromopyruvate ($V_i/V_0 = 0.68$). The linear semilog plots (solid lines) are consistent with a single pseudo-first-order process. The nonlinear plots (dashed lines) are theoretical curves obtained for two parallel pseudo-first-order reactions occurring at different rates as described in the text.

Therefore, DTNB as well as a second nonspecific reagent NEM were used as titrants in the following experiments to determine the number and reactivity of "essential" SH groups on malic enzyme which remained after alkylation by a variety of reagents.

"All-of-the-Sites" vs. "Half-of-the-Sites" Reactivity. The titration behaviors of E_1 and E_2 which have been previously inactivated by bromopyruvate, with DTNB and NEM, are shown in Figures 2 and 4. The kinetic behavior of SH groups (Figure 4) will be discussed subsequently. As indicated in Figure 2, the number of SH groups (3.8–4.1) on the fully active enzyme decreased with increasing inactivation; extrapolation of the linear plots to complete inactivation yielded 2.0 DTNB reactive (Figure 2A) and 1.86 [^{14}C]NEM-reactive (Figure 2B) groups per tetramer. These results corroborated the previously reported "half-of-the-sites" reactivity of bromopyruvate, which was shown to affect complete inactivation by alkylating 1.95 ± 0.1 class II SH groups (Chang & Hsu, 1977).

The SH groups of E_1 which have been previously inactivated to different extents by IAA, IAM, or NEM were titrated with DTNB. The results plotted as above were also linear (Figure 3). These reagents, however, showed "all-of-the-sites" reac-

TABLE I: Pseudo-First-Order Rate Constants of the "Fast" and "Slow" SH Groups on the Active and Bromopyruvate-Inactivated Malic Enzymes.[b]

titrant	enzyme	no. SH sites	k_1[a] (min^{-1})	k_1'[a] (min^{-1})	k_1/k_1'
[^{14}C]NEM	active E_2	4.2 ± 0.1	0.065 ± 0.005 (0.130)		
	completely inactivated E_2	2.0 ± 0.1		0.018 ± 0.005 (0.036)	3.6
DTNB	active E_1	4.2 ± 0.1	0.170 ± 0.005 (0.493)		
	completely inactivated E_1	2.0 ± 0.1		0.070 ± 0.005 (0.203)	2.4

[a] Numbers in parentheses are second-order rate constants in mM^{-1} min^{-1}. [b] Details of these experiments are described in the Figure 4 legend.

tivity, whereby complete inactivation resulted from the alkylation of four SH groups.

Kinetics of DTNB and [^{14}C]NEM Titration. The progress curves of the reaction of fully active E_1 (or E_2) with DTNB (or NEM) followed pseudo-first-order kinetics, as indicated by linear semi-log plots of SH groups remaining ($[SH]_r$) vs. time (Figure 4, open circles). The pseudo-first-order rate constants (k_1) were calculated from the slopes of these plots according to the equation: $\ln[SH]_r = -k_1 t + \ln [SH]_t$ (Table I). Linear kinetic plots were also obtained for the DTNB titration of malic enzyme (E_1) which has been partially inactivated by IAA, IAM, or NEM as described by experiments shown in Figure 3. The first-order rate constants of DTNB for the IAA-, IAM-, and NEM-inactivated enzymes were identical with each other and to the value for the reaction of DTNB with the fully active enzyme, and the average value from 27 separate determinations was 0.17 ± 0.005 min^{-1}. The corresponding second-order rate constant was 0.49 ± 0.03 mM^{-1} min^{-1}. The time course of reaction between E_1 or E_2 which has been labeled with bromopyruvate, with DTNB or [^{14}C]NEM as shown in experiments described in Figure 2 is presented in Figure 4. The completely inactivated enzyme (squares) also gave linear pseudo-first-order plots. However, the first-order rate constants were decreased from values of 0.065 ± 0.005 min^{-1} with NEM and 0.170 ± 0.005 min^{-1} with DTNB for the fully active enzyme to values of 0.018 ± 0.005 min^{-1} and 0.070 ± 0.005 min^{-1}, respectively (Table I). This 2.4–3.6-fold decrease in the observed rate constants reflected changes in the reactivity of SH groups from the "fast" reacting class of the active enzyme to the "slow" reacting class of the inactivated, dialkylated enzyme.

The kinetics of the enzyme partially inactivated by bromopyruvate were more complex, as indicated by the biphasic progress curves obtained for its reaction with NEM and DTNB (triangles in Figure 4). Since this enzyme presumably contained a mixture of SH groups in the "fast" and "slow" classes, the biphasic patterns were analyzed on the basis of two parallel pseudo-first-order reactions according to the equation $[SH]_r = ne^{-k_1 t} + me^{-k_1' t}$. The pseudo-first-order rate constant and the number of SH groups in the "fast" class were denoted as k_1 and n, respectively; whereas the corresponding values for the "slow" class of SH groups were denoted as k_1' and m. For the purpose of this calculation, k_1 was represented by the rate constant of the active enzyme control, and k_1' was represented by the rate constant of the completely inactivated enzyme (Table I). The number of SH groups in the "fast" and "slow" classes was selected from possible combinations where $n + m$ = SH groups present on the enzyme sample, to give the "best fit" theoretical plots. The biphasic curvature of the DTNB plot (Figure 4B) was less prominent than the NEM plot (Figure 4A), owing to the more limited separation of rate constants (Table I).

In Figure 5, the numbers of "fast" and "slow" SH groups determined by the above method were plotted against pyruvate incorporation. The linear plots clearly demonstrate that incorporation of pyruvyl residues was accompanied by simultaneous formation of an equal amount of "slow" groups up to a maximum of two per tetramer, and the disappearance of twice the amount of "fast" groups from 4.2 for the active enzyme to 0.2 for the completely inactivated, dialkylated enzyme.

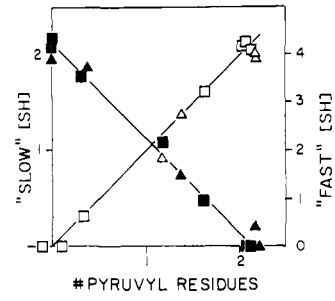

FIGURE 5: Correlation pf pyruvate incorporation with changes in the number of "fast" and "slow" SH groups. The titrants were: (triangles) [^{14}C]NEM; (squares) DTNB. The number of "fast" and "slow" SH groups is indicated by filled and open symbols, respectively. Pyruvate incorporation is obtained as the difference of the number of SH groups on the active enzyme and bromopyruvate-inactivated samples.

Discussion

In a previous study, the 36–40 DTNB-sensitive SH groups on malic enzyme have been resolved by us into three classes on the basis of their reactivity toward this reagent (Tang & Hsu, 1974). The inactivation of oxidative decarboxylase by DTNB followed typical bimolecular behavior, accompanied by the reaction of four class II SH groups. The inactivation of this enzyme by the substrate analogue bromopyruvate, however, showed saturation kinetics characteristic of an active-site directed process (Chang & Hsu, 1973), with complete inactivation occurring after the incorporation of pyruval residues on two of the four potentially available SH groups (Chang & Hsu, 1977). These results suggested the presence of four independent SH groups on the malic enzyme tetramer which react with DTNB in an "all-of-the-sites" manner, but which react asymmetrically with bromopyruvate, yielding "half-of-the-sites" stoichiometry. Similar behavior has been observed on the reaction of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with alkylating reagents (Levitzki, 1974).

The availability of premodified malic enzymes with blocked "nonessential" SH groups allowed us to study the stoichiometry and kinetic behavior of its "essential" SH groups. The enzymatically active derivatives contain four SH groups which exhibited "all-of-the-sites" behavior toward NEM as well as DTNB (Figure 1). The second-order rate constants obtained from the time course of SH group disappearance (Figure 4) were respectively 0.13 and 0.493 mM^{-1} min^{-1} (Table I), in

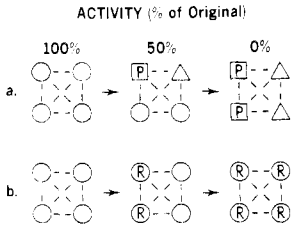

FIGURE 6: Schematic representation of the conformational states of malic enzyme. (A) "Half-of-the-sites" reaction with bromopyruvate (P). (B) "All-of-the-sites" behavior of DTNB, IAA, IAM, and NEM (R). The four subunits (circles) of the tetramer react with these reagents as a single set. These subunits are identical in size. Each binds a molecule of NADPH or $NADP^+$ independently and equivalently.

good agreement with corresponding values of 0.134 and 0.476 mM^{-1} min^{-1} for the class II SH groups obtained from inactivation parameters (Tang & Hsu, 1974). IAA and IAM were also shown to be "all-of-the-sites" reagents, reacting with all four SH groups and causing concomitant inactivation (Figure 3). Our finding that partial alkylation of malic enzyme by IAA, IAM, and NEM did not affect its kinetic behavior toward DTNB is also consistent with this interpretation.

The disappearance of two DTNB (or NEM) titratable SH groups on the enzyme following complete inactivation by bromopyruvate is in accord with the previously observed "half-of-the-sites" reactivity based on [^{14}C]pyruvate incorporation data (Chang & Hsu, 1977).

The reaction kinetics of the four SH groups in the fully active malic enzyme derivatives with either DTNB or NEM can be accounted for satisfactorily as a single pseudo-first-order process. In the enzyme completely inactivated by bromopyruvate, the two remaining SH groups react with DTNB or NEM in a pseudo-first-order manner, but at rates 2.4- and 3.6-fold slower than the unalkylated enzyme. In the partially inactivated enzyme with fewer than two pyruvyl residues incorporated per tetramer, the reaction of SH groups could not be described by pseudo-first-order kinetics (Figures 4A and 4B). The biphasic behavior could be accounted for by assuming that there were two parallel pseudo-first-order reactions, with the fast reaction having a rate constant corresponding to that determined for the unalkylated enzyme and the slow reaction having a rate constant equal to that for the completely alkylated enzyme containing two pyruvyl residues per tetramer (Table I). With these two measured rate constants, it is possible to analyze a number of partially inactivated enzyme samples containing variable amounts of bound pyruvate. The number of SH groups in the "slow" class increased quantitatively with bound pyruvyl residues, whereas the number of SH groups in the "fast" class decreased in amount equal to the sum of the "slow" and alkylated groups (Figure 5).

Although the biphasic kinetic plots (Figure 4) are satisfactorily explained in terms of two classes of SH groups, this determination cannot exclude the possibility of a larger number of species with rate constants too close to be resolved. The SH groups of yeast glyceraldehyde-3-phosphate dehydrogenase, which showed negative cooperativity between subunits, have been resolved into three classes on the basis of their kinetic reactivity (Stallcup & Koshland, 1973).

Since the four SH groups on the fully active enzyme (one residing in each subunit) react stoichiometrically and equivalently toward nonspecific reagents, the lower rate observed for the reaction of those groups remaining on the bromopyruvate-inactivated enzyme with the same reagents therefore indicates a change in their reactivity induced by the affinity label. This behavior would also account for the "half-of-the-sites" reactivity; however, the possibility of preexisting asymmetry at substrate sites which is recognizable only by the affinity label is unlikely, but cannot be excluded by these experiments. The "half-of-the-sites" reactivity appears to be associated with the pyruvate-like structure of the bromopyruvate molecule. Binding of this reagent at the substrate site promotes specific conformation changes which are transmitted across the subunit boundary, resulting in decreased accessibility of the SH group on the unoccupied subunit neighbor. Alternatively, an explanation based on steric hindrance and/or charge repulsion by the enzyme-bound ligand seems unlikely. Reagents structurally unrelated to the substrate, but similar to bromopyruvate in charge (DTNB, IAA) or size (IAA, IAM), or larger than bromopyruvate (DTNB, NEM), are unable to induce this response. Moreover, while the "half-of-the-sites" labeled enzyme does not react with bromopyruvate, it is still accessible to bulkier reagents such as DTNB and NEM (Figure 2).

It should be noted that changes in reactivity represent a sensitive indicator of conformation changes, as shown by the elegant work of Blackburn & Schachman (1977) on aspartate transcarbamoylase. The observed "half-of-the-sites" behavior of malic enzyme and the attendant reduction in the reactivity of SH groups with nonspecific reagents may be interpreted in terms of the concept of ligand-induced negative cooperativity (Koshland et al., 1966), as indicated by the minimum model shown in Figure 6. The reaction with "all-of-the-sites" reagents is also given as comparison. The subunits have equal reactivity toward these reagents, indicating "functional symmetry." The tetramer behaves as a dimer-of-dimers[3] in its reaction with bromopyruvate. Conformation changes induced by the binding of this ligand to the first subunit turn off the adjacent subunit, inactivating its catalytic site for the reaction with malate, as well as converting the initially reactive "fast" SH group into the unreactive "slow" SH group. The catalytic activities on malate decarboxylation are 100%, 50%, and 0% for the unalkylated, monoalkylated, and dialkylated enzymes, respectively.

Schmerlik et al. (1977) showed that the dissociation of NADPH is rate-limiting in the oxidative decarboxylation of L-malate. If all four active-sites are operating simultaneously, a "full-size" burst of enzyme-bound NADPH would be seen. However, in transient kinetic studies (Reynolds et al., 1978), we obtained a burst-size which was only approximately half of the active-sites concentration. "Half-of-the-sites" reactivity in the catalytic reaction would be consistent with the presence of two "tight" Mn^{2+} binding sites, the apparent kinetic negative cooperativity of Mn^{2+} (Hsu et al., 1976), and the observed half-site stoichiometry of alkylation by bromopyruvate. The decreased reactivity of SH groups on the bromopyruvate-inactivated enzyme documented in this paper lends further support to this interpretation. The cooperative behavior of malic enzyme has also been detected by fluorescence titration experiments which gave two K_D values of 17 μM and 500 μM for L-malate in the E·Mn^{2+}·NADPH·malate complex (Hsu et al., 1976). It is conceivable, therefore, that the active sites alternate during the catalytic cycle, possibly by a "flip-flop" type of mechanism described by Lazdunski (1974).

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Relation between Calcium Requirement, Substrate Charge, and Rabbit Polymorphonuclear Leukocyte Phospholipase A_2 Activity†

Richard Franson* and Moseley Waite

ABSTRACT: Phospholipase A_2 isolated from rabbit granulocytes hydrolyzes liposomes of synthetic phosphatidylethanolamine and the phospholipids of autoclaved *E. coli* optimally at pH 7.4 in the presence of 1 mM of Ca^{2+}. EDTA is a potent inhibitor of phospholipase A_2 activity; Mg^{2+} does not substitute for Ca^{2+} in this reaction. Phospholipase A_2 activity in the presence of 1 mM of Ca^{2+} is optimal when the surface charge of phosphatidylethanolamine liposomes is slightly negative (−2.6). Concentrations of Ca^{2+} greater than 1 mM inhibited enzymatic activity and increased the surface charge (ζ potential) of phosphatidylethanolamine liposomes. The amphipaths, cetyltrimethylammonium bromide and diacetyl phosphate, altered the surface charge of phosphatidylethanolamine liposomes and inhibited phospholipase A_2 activity. The inhibition was partially reversed by the addition of the oppositely charged amphipath and was not due to detergent effects of the amphipaths since phospholipase A_2 activity was unaffected by similar concentrations of hexadecanol and Triton X-100. When the surface charge deviated from the proper range, maximal hydrolysis of phosphatidylethanolamine occurred at concentrations of Ca^{2+} that were strongly inhibitory in the absence of the amphipaths. These data demonstrate that the granulocytic phospholipase A_2 has an absolute catalytic requirement for calcium; higher concentrations of Ca^{2+} inhibit enzymatic activity in part by altering the surface charge of the substrate liposome.

Hydrolysis of a smectic mesophase of phospholipid by phospholipase A is influenced by the physical nature of the substrate liposome (Bangham, 1972; Brockerhoff & Jenson, 1974; Dawson, 1964), particularly the packing or spatial arrangement of phospholipids and the surface charge or ζ potential of the liposome at the lipid-water interface. Many studies have demonstrated that packing of phospholipid within bilayers or monolayers affects the susceptibility of phospholipids to hydrolysis by phospholipases. However, the question of whether phospholipases preferentially hydrolyze substrates with a given surface charge remains unanswered. Goldhammer et al. (1975) have reported that the electrokinetic characteristics of liposomes are not important determinants of susceptibility to phospholipase A attack, whereas other reports (Bangham & Dawson, 1958; Dawson et al., 1976) have shown that the electrophoretic charge of the substrate affects the hydrolysis of liposomes by lecithinase and phospholipase C. This report examines the electrokinetic substrate requirements of a highly cationic PLA_2[1] isolated from rabbit granulocytes in an attempt to separate the role played by Ca^{2+} in the catalytic event from its influence on the surface charge of the substrate liposome.

Experimental Procedure

Materials. The phospholipids of *E. coli* were labeled during growth with [1-^{14}C]oleate and the bacteria were autoclaved to destroy bacterial phospholipases and to render the bacterial phospholipids more susceptible to hydrolysis (Patriarca et al., 1972; Franson et al., 1974, 1978). 1-Acyl-2-[1′-^{14}C]linoleoyl-3-glycerophosphorylethanolamine and 1-acyl-2-[1′-^{14}C]linoleoyl-3-glycerophosphorylcholine were synthesized according to the method of Waite & van Deenen (1967). CTMB was bought from Eastman Kodak Co., Rochester, N.Y., and hexadecanol, Triton X-100, and DCP were pur-

† From the Medical College of Virginia, Department of Biophysics, Richmond, Virginia 23298, and Bowman Gray School of Medicine, Department of Biochemistry, Winston-Salem, North Carolina 27103. *Received April 14, 1978.* This research was supported by National Institutes of Health Grants HL-21116, HL-19148, AM 11799, and AM 17392.

[1] Abbreviations used: PLA_2, phospholipase A_2; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CTMB, cetyltrimethylammonium bromide; DCP, diacetyl phosphate.

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