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# *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System. Functional Asymmetry in Enzyme I Subunits Demonstrated by Reaction with 3-Bromopyruvate†

Henk Hoving, Ria ten Hoeve-Duurkens, and George T. Robillard*

ABSTRACT: In the bacterial phosphoenolpyruvate-dependent sugar transport systems, enzyme I ($E_I$) is responsible for the initial reaction step which is the transfer of the phosphoryl group from phosphoenolpyruvate to a cytoplasmic phosphocarrier protein (HPr). The inactivation of enzyme I by the substrate analogue 3-bromopyruvate has been investigated. Incubation of enzyme I with only micromolar concentrations of this reagent results in complete and irreversible loss of enzymatic activity within a few minutes. Other alkylation reagents such as 2-iodoacetate, 3-bromopropionate, or 5-bromovalerate are far less effective inhibitors of enzyme I, suggesting that the inactivation by 3-bromopyruvate is brought about by the alkylation of one or more essential residues at the active site. Phosphoenolpyruvate and pyruvate, or phosphoenolpyruvate and oxalate, when added together, protect against inactivation by bromopyruvate. Experiments with bromo[2-$^{14}$C]pyruvate showed that one residue per enzyme I dimer is first alkylated without causing any loss of enzymatic activity. Alkylation of a second residue causes complete inactivation. Both alkylated residues are cysteines. The observations in this report together with published data on the phosphorylation of $E_I$ suggest that the subunits of the $E_I$ dimer modulate one another's activity during the turnover of the enzyme.

Enzyme I, a component of the bacterial phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phosphocarrier protein (HPr). This is the first step in a series that ultimately leads to the phosphorylation and concomitant transport of hexoses and hexitols into the bacterial cell [for recent reviews, see Hays (1978) and Robillard (1982)]. The enzyme I catalyzed reaction proceeds via a phosphoenzyme I intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981; Weigel et al., 1982). Even though the activity of enzyme I requires a dimeric form of the enzyme, only one phosphoryl group is bound per dimer (Hoving et al., 1981; Misset & Robillard, 1982). These two observations are consistent with negative cooperative interactions or a flip-flop mechanism.

3-Bromopyruvate has been used successfully for active-site labeling of a large number of PEP- and pyruvate-utilizing enzymes (Meloche, 1965, 1967; Barnett et al., 1971; Berghauser et al., 1981; Hudson et al., 1975; Chang & Hsu, 1977; Yoshida & Wood, 1978; Kameshita et al., 1979; Yon & Suelter, 1979). Saier et al. (1980) have reported inactivation of enzyme I by 3-bromopyruvate. This paper presents studies using 3-bromo[2-$^{14}$C]pyruvate to specify the number of amino acid residues involved in the inactivation of enzyme I by this substrate analogue and to identify these residues. The object of these studies was to determine whether the asymmetry found in the phosphorylation sites also occurs at the level of the catalytically active base(s).

## Materials and Methods

*Chemicals and Enzymes.* [1-$^{14}$C]PEP (monocyclohexylammonium salt, sp act. 12 $\mu$Ci/$\mu$mol) and [2-$^{14}$C]pyruvate (sodium salt, sp act. 15.8 $\mu$Ci/$\mu$mol) were purchased from Amersham. Nonradioactive pyruvate (sodium salt), PEP (monocyclohexylammonium salt), 3-bromopyruvic acid, 3-

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[1] Abbreviations: *S*-CHE-cysteine, *S*-(carboxyhydroxyethyl)cysteine; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; DTT, dithiothreitol; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; Ox, oxalate; Pyr, pyruvate; BrPyr, 3-bromopyruvate.

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bromopropionic acid, and 5-bromovaleric acid were purchased from Sigma Chemical Co. 2-Iodoacetic acid was purchased from Boehringer. Enzyme I was purified from *Escherichia coli* P650 as described previously (Robillard et al., 1979; Brouwer et al., 1982). The purified enzyme was stored in a 25 mM sodium phosphate buffer, pH 7.2, containing 0.5 mM DTT and 1 mM $NaN_3$ at −20 °C. Enzyme I concentrations were determined by measuring the initial burst of [$^{14}$C]pyruvate from [$^{14}$C]PEP upon the addition of enzyme I (Brouwer et al., 1982). The enzyme I concentrations given in the text are always concentrations of available phosphoryl group binding sites and correspond to dimer concentrations (Misset & Robillard, 1982).

*Preparation of 3-Bromo[2-$^{14}$C]pyruvic Acid.* 3-Bromo[2-$^{14}$C]pyruvic acid was prepared from [2-$^{14}$C]pyruvate basically as described by Meloche (1967); 3.2 μmol of sodium [2-$^{14}$C]pyruvate and 20 μmol of nonradioactive sodium pyruvate were dissolved in 140 μL of glacial acetic acid. A slight excess of bromine over pyruvate (20% excess) was added, together with 25 μL of concentrated sulfuric acid as a catalyst. The reaction mixture was allowed to reflux at 55 °C for 1 h. The very pale yellow solution was frozen and stored at −20 °C and was used without further purification. Bromopyruvate appeared to be stable for more than 10 months when stored under these conditions. The concentration of bromopyruvate in the reaction mixture was determined by means of the LDH-catalyzed reduction to bromolactate with NADH (Berghauser et al., 1971). These assays were carried out in a 200 mM potassium phosphate buffer, pH 7.3, containing 0.3 mM NADH. The bromolactate solution was diluted 600 times in this assay solution, and the extinction at 340 nm was read before and 10 min after the addition of 0.1 mg/mL LDH. The decrease of the extinction was shown to be due to the reduction of bromopyruvate, and not the remaining pyruvate or 3-hydroxypyruvate, by repeating the assay with a bromopyruvate solution to which excess DTT was added first. No decrease in NADH extinction was observed in this case, showing that all the substrate for LDH was removed and hence must have been bromopyruvate. Before use in enzyme I inactivation experiments, the strongly acidic bromopyruvate solution (142 mM bromopyruvate in acetic acid) was diluted 40 times in 1 M sodium phosphate, pH 9, to give a stock solution at pH 6.0.

*Sugar Phosphorylation Assay.* Enzyme I activities were measured in a sugar phosphorylation assay by using a crude cell extract of an enzyme I lacking mutant of *Salmonella typhimurium* (strain SB 1690). The assay is based on the overall PTS reaction, the phosphorylation of α-methylglucose from PEP. The experimental procedure has been described by Misset et al. (1980). In this assay, both half-reactions of enzyme I are of importance: phosphorylation of enzyme I from PEP and the transfer of this phosphoryl group to HPr.

*PEP–Pyruvate Phosphoryl Group Exchange Assay.* This assay is based on the enzyme I catalyzed interconversion of PEP and pyruvate, via phosphoenzyme I, at chemical equilibrium. All assays were carried out in a 25 mM sodium phosphate buffer, pH 7.2, containing 10 mM $MgCl_2$, 1 mM DTT, and 1 mM $NaN_3$, at 37 °C, by using 6.25 μM nonradioactive PEP and 900 μM pyruvate. The assays were started by the addition of 1.25 μM [1-$^{14}$C]PEP, and the isotope exchange was monitored as described previously (Hoving et al., 1981).

## Results

*Inactivation of Enzyme I by 3-Bromopyruvate.* 3-Bromopyruvate is a very potent inhibitor of enzyme I: incubation of 0.25 μM enzyme I (containing 2.5 μM DTT) with 10 μM bromopyruvate in a 25 mM sodium phosphate buffer, pH 8.0 at 37 °C, resulted in irreversible and complete loss of enzymatic activity with $t_{1/2}$ = 1 min. At pH 7.0, the inactivation is approximately 15 times slower (data not shown). Other alkylating reagents proved to be far less effective inhibitors of enzyme I. Incubation of 0.25 μM enzyme I (containing 2.5 μM DTT) with 100 μM 2-iodoacetate, 3-bromopropionate, or 5-bromovalerate for 15 min at pH 8.0 resulted in 100%, 75%, and 100% remaining activity, respectively. The enzyme I activities were measured in a sugar phosphorylation assay (see Materials and Methods). These findings seem to be in accordance with preliminary data reported by Saier et al. (1980).

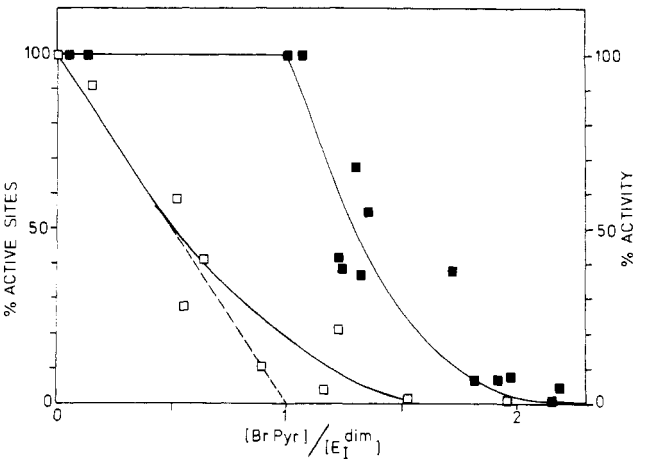


FIGURE 1: Enzyme I activity and enzyme I phosphoryl group binding sites as a function of the number of 3-bromo[2-$^{14}$C]pyruvate labels incorporated per enzyme I dimer ([BrPyr]/[$E_I^{dim}$]). (■) 1.7 μM enzyme I + 50 μM DTT incubated with 228 μM 3-bromo[2-$^{14}$C]-pyruvate in 25 mM sodium phosphate buffer, pH 6.8, containing 10 mM $MgCl_2$ for 0.3–5 min at 37 °C. The reaction was stopped by the addition of 2.7 mM DTT. Samples of 200 μL were applied to a Sephadex G-25 column (1 × 35 cm, elution rate 25 mL/h) to determine the extent of labeling. Enzyme I activities were measured as pseudo-first-order rate constants for the interconversion of PEP and pyruvate at chemical equilibrium, using 13 nM enzyme I. (□) 1.2 μM enzyme I + 25 μM DTT incubated with 112 μM 5-bromovalerate in 25 mM sodium phosphate buffer, pH 7.9, containing 10 mM $MgCl_2$ for 15 min at 37 °C. 11 μM 3-bromo[2-$^{14}$C]pyruvate was then added, and the reaction was allowed to proceed for 1–15 min at the resulting pH of 7.7 at 37 °C. The reaction was stopped by the addition of 1.1 mM DTT. The extent of labeling was determined as described above. The number of residual phosphoryl group binding sites was determined by adding 1.8 μM [2-$^{14}$C]PEP to the reaction mixture and measuring the amount of [$^{14}$C]pyruvate formed.

*Stoichiometry of Labeling with 3-Bromopyruvate.* A series of experiments with 3-bromo[2-$^{14}$C]pyruvate were carried out in order to determine the number of amino acid residues involved in the inactivation of enzyme I. Since the enzyme is purified and stored in the presence of DTT, an excess of bromopyruvate over DTT thiol groups had to be employed to obtain inactivation of enzyme I. In order to detect the enzyme-bound label after inactivation, it was necessary to use 1–2 μM enzyme I containing 30–60 μM DTT. Therefore, 228 μM bromopyruvate was used in these experiments. The reaction with enzyme I was terminated by the addition of excess DTT. Remaining enzyme I activity was measured in the PEP–pyruvate phosphoryl group exchange assay (see Materials and Methods). The enzyme was separated from free label by gel filtration on a Sephadex G-25 column, and the stoichiometry of the labeling was calculated from the specific radioactivity of bromopyruvate and the enzyme concentration.

The enzyme I activity remaining after reaction with bromopyruvate is plotted vs. the number of labels incorporated

per dimer in Figure 1. The data (closed symbols) show that one label is incorporated per dimer without causing any loss of enzymatic activity, whereas labeling of a second residue results in complete inactivation. The first residue is fully alkylated within 20–30 s, and the alkylation of the second residue takes place in approximately 5 min. No labeling and 100% activity were found when enzyme I was (a) incubated at pH 6.8 and 37 °C for 5 min in the presence of 2.7 mM DTT and 228 μM bromopyruvate (DTT added first) and (b) incubated under the same conditions, but without DTT and bromopyruvate.

As mentioned previously, no inactivation of enzyme I was observed upon incubation with excess 5-bromovalerate over DTT thiol groups. Nevertheless, the reagent does react with enzyme I. The open symbols in Figure 1 represent experiments in which enzyme I was incubated with excess bromovalerate over DTT thiol groups prior to the addition of a much lower concentration of bromopyruvate. The number of remaining enzyme I phosphoryl group binding sites was determined from the initial burst of [$^{14}$C]pyruvate formed from [$^{14}$C]PEP upon the addition of enzyme I (Brouwer et al., 1982). This quantity is plotted vs. the number of bromopyruvate labels incorporated per dimer in Figure 1 (open symbols). The data point with 100% phosphoryl group binding sites represents enzyme I samples which were (a) incubated with 112 μM bromovalerate for 15 min at pH 7.9 and 37 °C and subsequently with 1.1 mM DTT + 11 μM bromopyruvate for 15 min at pH 7.7 and 37 °C (DTT added before bromopyruvate), (b) incubated with 112 μM bromovalerate for 30 min at pH 7.9 and 37 °C, and (c) not pretreated. The data show that the treatment with bromovalerate does not affect the number of phosphoryl group binding sites on enzyme I and that upon the subsequent addition of bromopyruvate one phosphoryl group binding site is lost for every one molecule of bromopyruvate bound. Similar results were obtained when the enzymatic activity, as measured kinetically in the PEP–pyruvate phosphoryl group exchange assay, was plotted. These results indicate that the amino acid residue which was rapidly alkylated by bromopyruvate in the absence of bromovalerate is now alkylated by bromovalerate, again without causing any loss of enzymatic activity. In summary, Figure 1 shows that enzyme I, in the absence of substrate or products, possesses two sites per dimer which can be alkylated. The first site is the more reactive of the two, but the second site is the more selective of the two.

*Dimerization Properties of Alkylated Enzyme I.* Enzyme I is a dimer in the active form. All evidence suggests that the subunits are identical. When enzyme I is assayed at different enzyme concentrations, a more than proportional increase of the measured activity with the total enzyme concentration is normally observed in the concentration range used in the experiments presented here. This nonlinear behavior has been ascribed to the reversible dissociation of active enzyme I dimers into inactive monomers (Waygood et al., 1979; Misset et al., 1980; Hoving et al., 1981). Since only one phosphoryl group is incorporated per dimer and since complete activity is lost during the alkylation of a single residue per dimer, it would appear that, in the dimer form, the protomers become asymmetric upon reaction at one of the two subunits. In order to determine whether alkylation of the first site alters the association behavior of the subunits, the concentration dependence of the activity of native enzyme I and singly labeled enzyme I was compared. The activity of unlabeled enzyme I and of enzyme I containing one bromopyruvate label per dimer is plotted vs. the total enzyme concentration in Figure 2. The fact that both enzyme preparations exhibit the same nonlinearity in this plot demonstrates that the dissociation constants of singly labeled and unlabeled dimers are equal.

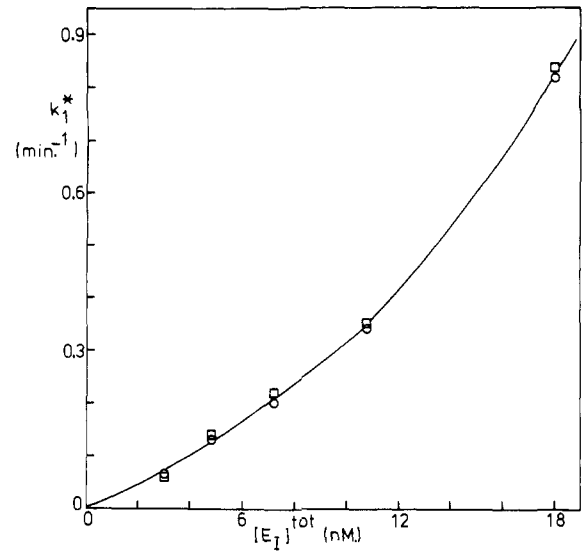


FIGURE 2: Pseudo-first-order rate constant for the enzyme I catalyzed interconversion of PEP and pyruvate at chemical equilibrium as a function of the total enzyme concentration. Experimental conditions were the following: 7.5 μM PEP and 900 μM pyruvate in 25 mM sodium phosphate buffer, pH 7.2, containing 10 mM $MgCl_2$ 1 mM DTT, and 1 mM $NaN_3$ at 37 °C. (○) Enzyme I with one residue per dimer alkylated with 3-bromopyruvate; (□) unlabeled enzyme I.

In the previous sections, the PEP–pyruvate phosphoryl group exchange assay was used to detect inactivation of enzyme I, because it focuses on the first phosphoryl group transfer step (i.e., transfer from PEP to enzyme I). Detection of inactivation in the sugar phosphorylation assay, which depends on both enzyme I catalyzed phosphoryl group transfer steps (i.e., transfer from PEP to enzyme I and subsequently to HPr), would have left the question unanswered as to whether the first or the second step is blocked. An enzyme which is still active in the PEP–pyruvate phosphoryl group exchange assay could be inactive in the sugar phosphorylation assay, if the phosphoryl group transfer from phophoenzyme I to HPr were blocked. Therefore, the activity of the enzyme containing only one bromopyruvate label per dimer and exhibiting 100% activity in the PEP–pyruvate phosphoryl group exchange assay was also checked in the sugar phosphorylation assay, and 100% activity was found again. Enzyme I which was treated with bromovalerate was also shown to be fully active in this assay (see above). Thus, it can be concluded that alkylation of one amino acid residue per enzyme I dimer, either by bromopyruvate or by bromovalerate, results in enzyme I that has unchanged dissociation properties and unchanged dimer activity. Alkylation of a second residue, which seemes to be inaccessible to bromovalerate, causes complete loss of enzymatic activity.

*Influence of Substrate and/or Products on the Inactivation and Degree of Labeling.* When substrate or product protects against inactivation, it supports the conclusion that the residue being modified is in the region of the catalytic site. The data in Table I list the effects of various compounds on the rate of inactivation of $E_I$ by bromopyruvate. PEP (100 μM) or pyruvate (10 mM) alone does not protect significantly. However, the addition of 100 μM PEP plus 10 mM pyruvate in the presence of 10 mM $MgCl_2$ did result in protection against inactivation by bromopyruvate: 30% activity remained after 10 min, and with 5-fold higher PEP and pyruvate concentrations, no loss of activity was observed within 10 min.

Table I: Influence of PEP, Pyruvate, and Oxalate on the Rate of Inactivation of $E_I$ by 200 $\mu$M Bromopyruvate[a]

| incubation time (min) | % of remaining activity | | | | | | |
|---|---|---|---|---|---|---|---|
| | no additions | 100 $\mu$M PEP | 10 mM Pyr | 100 $\mu$M PEP + 10 mM Pyr | 1 mM Ox | 10 mM Ox | 100 $\mu$M PEP + 1 mM Ox |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.5 | 100 | | | 100 | | | |
| 1 | 50 | | | | 70 | | 100 |
| 5 | 10 | 10 | 10 | | 10 | 70 | 100 |
| 10 | 0 | 0 | 0 | 30 | | | |
| 15 | | | | | 0 | 0 | 80 |

[a] Incubation was carried out as stated in the legend to Figure 1. The exchange activity was assayed as described under Materials and Methods.

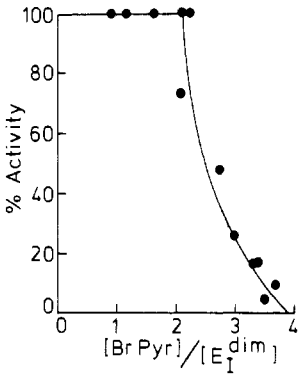


FIGURE 3: Enzyme I activity as a function of the number of 3-bromo[2-$^{14}$C]pyruvate labels incorporated per enzyme I dimer ([BrPyr]/[$E_I^{dim}$]). 1.6 $\mu$M $E_I$ and 50 $\mu$M DTT were incubated with 194 $\mu$M 3-bromopyruvate in 25 mM sodium phosphate buffer, pH 6.8, containing 10 mM $MgCl_2$, 1 mM oxalate, and 100 $\mu$M PEP. Details of the procedures used to determine the activity and extent of labeling are given in the legend to Figure 1.

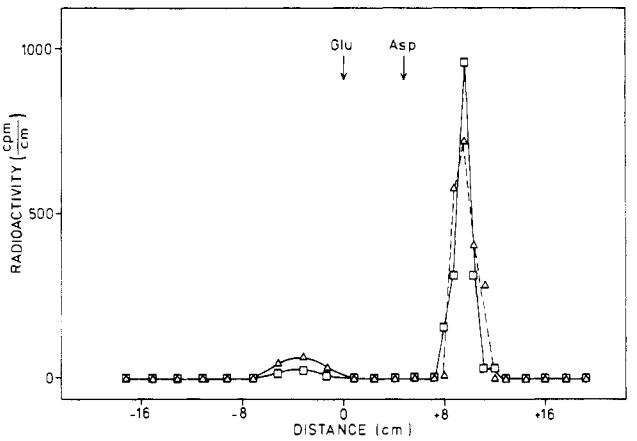


FIGURE 4: Paper electrophoretogram of *S*-CHE-cysteine from 3-bromo[2-$^{14}$C]pyrutate-labeled and hydrolyzed glutathione (□) and of 3-bromo[2-$^{14}$C]pyruvate-labeled and hydrolyzed enzyme I (△). Electrophoresis was carried out on Whatman 3MM paper (25 × 57 cm) in pyridine/acetic acid/water (1/10/290), pH 3.5, for 45 min at 3000 V and 200 mA. After the paper was dried it was cut into 0.8-cm strips, and the radioactivity was measured in a Packard emulsifier–scintillator. Asp and Glu were detected as ninhydrin-positive spots. The cathode is at the left-hand side.

Oxalate, an analogue of enolpyruvate, protects more efficiently than pyruvate. Maximal protection, however, occurs in the presence of PEP plus oxalate. Under these conditions, enzyme I should be in a P–$E_I$/oxalate complex form. The correlation between the number of labels incorporated and the loss of activity is shown in Figure 3. Two moles of label is incorporated per dimer without loss of activity in the presence of 1 mM oxalate. Incorporation of the third mole of label results in inactivation. In the absence of oxalate (Figure 1), 1 mol of label was incorporated per dimer without loss of activity, and incorporation of a second mole of label resulted in complete inactivation.

*Identification of the Labeled Residues.* Enzyme I was inactivated with 3-bromo[2-$^{14}$C]pyruvate as described in Figure 1 (closed symbols). The stoichiometry of the labeling was 2 per dimer. The inactivated enzyme (14 mL, 1.7 $\mu$M) was dialyzed overnight against a 200-fold excess of $H_2O$, lyophilized, dissolved in 1.4 mL of $H_2O$, and chromatographed on a Sephadex G-25 column (1 × 35 cm, elution rate 25 mL/h) in 50 mM ammonium bicarbonate, pH 7.9, to remove the free label. The labeled enzyme was pooled, reduced with 2 mM sodium borohydride, lyophilized, taken up in 6 N HCl, and hydrolyzed in sealed, evacuated tubes at 110 °C for 24 h. After hydrolysis, the samples were dried at 50 °C under vacuum.

*S*-CHE-cysteine was prepared by alkylating glutathione with 3-bromopyruvate, basically as described by Meloche (1970). 3-Bromo[2-$^{14}$C]pyruvate (70 $\mu$M) was added to a 250 $\mu$M solution of glutatione in $H_2O$. The alkylation was allowed to proceed at room temperature, and all the bromopyruvate reacted within 15 min. Concentrations of bromopyruvate in the reaction mixture were determined by using the LDH-catalyzed reduction to bromolactate (see Materials and Methods). The reaction product was treated with sodium borohydride and was hydrolyzed as described above for the enzyme I samples.

The hydrolyzed enzyme I and glutathione samples were desalted by chromatography through a Sephadex G-10 column (0.7 × 90 cm) eluted with $H_2O$ (6 mL/h). All the radioactivity in both samples eluted as a broad peak (8 mL) just before the salt peak. According to Gelotte (1960), the amino acids Asp and Glu elute before the salt front on Sephadex columns when run in $H_2O$. Our results with alkylated and hydrolyzed glutathione and enzyme I show that *S*-CHE-cysteine and the labeled amino acids from enzyme I behave similarly, presumably because of the second carboxyl group introduced. The desalted samples were analyzed by means of paper electrophoresis at pH 3.5. The result is shown in Figure 4. The positions of Asp and Glu, the two amino acids that also contain a second carboxyl group, are indicated to illustrate the resolution of the technique. The exact overlap of the peaks in Figure 4 identifies the product from enzyme I as *S*-CHE-cysteine. The minor peaks which migrate toward the cathode were not identified. It is interesting to note that these peaks have also been reported by Hudson et al. (1975) in paper electrophoresis of *S*-CHE-cysteine at pH 5.5.

Since doubly labeled enzyme I was used, it can be concluded that both labeled residues are cysteines. Care must be taken with such a conclusion if not all the enzyme-bound radioactivity is retained during the preparation of the samples. A loss of radioactivity varying between 10 and 50% was always observed after the hydrolyzed samples of both enzyme I and glutatione were dried. According to Hartman (1977), this loss of radioactivity is indicative of the formation of sulfoxide from the cysteine thioether. The fact that this loss is observed for

the glutathione as well demonstrates that the experimental procedure causes 10–50% loss of *S*-CHE-cysteine and hence that all enzyme I bound radioactivity must also have been *S*-CHE-cysteine.

## Discussion

*Evidence for Active-Site Alkylation.* It is known from previous studies that the enzymatic protonation of PEP is an essential step in the phosphorylation of enzyme I (Hoving et al., 1981). In this paper, we show that enzyme I is rapidly inactivated by the substrate analogue 3-bromopyruvate. The following lines of evidence suggest that the inactivation involves alkylation at the active site, presumably alkylation of the base involved in the proton transfer step. (i) The inactivation by the substrate analogue 3-bromopyruvate is very rapid, whereas similar alkylating reagents that bear less resemblance to the natural substrate appeared to be far less effective inhibitors. Presumably, the bromomethyl group in 3-bromopyruvate is in an ideal position relative to the base that would abstract a proton from the pyruvate methyl group or donate a proton to the PEP methylene group in the normal reaction. (ii) The addition of concentrations of PEP and pyruvate which saturate the enzyme with its natural substrates (Hoving et al., 1981, 1982) resulted in a marked protection against inactivation by bromopyruvate. The fact that pyruvate or oxalate provides an optimal protection against bromopyruvate in the presence of PEP suggests that the formation of functional enzyme–substrate complexes is involved in this protection. (iii) Not only was the inactivation of enzyme I demonstrated in a kinetic assay but also it was shown that the inactivated enzyme cannot be phosphorylated from PEP (Figure 1, open symbols). A simple calculation using a second-order rate constant for the phosphorylation of enzyme I of $10^9$ $M^{-1}$ $min^{-1}$ (Hoving et al., 1982) shows that 98% of the active enzyme is phosphorylated in less than a second under the experimental conditions employed. Phosphorylation of enzyme I (i.e., pyruvate formation) was always measured after several minutes, and no phosphorylation was detected for the doubly alkylated enzyme. This demonstrates that the doubly alkylated enzyme is totally inactive and thus that at least one alkylated cysteine is an essential residue.

*Stoichiometry of Bromopyruvate Labeling and Enzyme I Subunit Composition.* The data presented in this paper show that alkylation of two cysteines per dimer is involved in the inactivation of enzyme I by bromopyruvate but that alkylation of the first one does not affect the enzymatic activity. Thus, either the first cysteine is not essential and only the second one is essential for enzymatic activity or both are essential residues but both must be alkylated to render the dimer inactive. In the latter case, one could envision that both subunits contain one such essential residues and that labeling on one subunit leaves the phosphoryl group binding site on the other subunit intact. The differential alkylation rates would then by indicative of a cooperative interaction.

Some evidence concerning whether both subunits are identical or not can be obtained from the differential alkylation rates and the dissociation behavior of the 1:1 labeled and still active dimers. It can be inferred from the data in Figure 2 that the dissociation constants of the 1:1 labeled and unlabeled dimers are equal. We know that under the conditions of the assay there is a dynamic equilibrium between monomers and dimers (Misset et al., 1980; Hoving et al., 1981, 1982). If the two subunits are identical, the equal dissociation constant would imply that the 1:1 labeled enzyme exists as a statistical mixture of unlabeled and singly and doubly labeled dimers. Therefore, it is difficult to explain why the labeling with bromovalerate or the rapid labeling with bromopyruvate should stop at only one label per dimer and not continue to yield 100% double labeled dimers. These data are much easier to explain for an enzyme possessing two nonidentical subunits. In this case, the enzyme I dimers are of the form AB, and the rapid labeling with bromopyruvate, or the labeling with bromovalerate, to yield still active dimers will take place on only one subunit. Since sequential dissociation and reassociation will always yield dimers of the form AB, all the dimers will now contain one alkylated cysteine. Such asymmetry is supported by the results of the labeling experiments in the presence of PEP and oxalate. In the absence of protecting compounds, incorporation of the second mole of label results in complete inactivation. PEP and pyruvate or oxalate protect the activity by preventing labeling at this site but, at the same time, cause another site which is not essential for activity to become labeled. Inactivation subsequently occurs with the alkylation of a single residue per dimer.

We have shown that when enzyme I is combined with excess [$^{14}$C]PEP, a burst of [$^{14}$C]pyruvate occurs which is a measure of the amount of P–$E_I$ formed (Brouwer et al., 1982). When [$^{32}$P]PEP is employed $^{32}$P-labeled enzyme I is produced which can be isolated by gel filtration or chromatography on DE-81 filter paper (Weigel et al., 1982). Both procedures give the same result; *E. coli* P–enzyme I carries one phosphoryl group per $E_I$ dimer. Thus, both our inactivation and our phosphorylation data are consistent with an enzyme I dimer containing only one functional active site.

Weigel et al. (1982) report that *S. typhimurium* enzyme I carries two phosphoryl groups per dimer, in contrast with our finding of one phosphoryl group per dimer for the *E. coli* enzyme I. Furthermore, they have reported an equilibrium constant for the phosphorylation reaction with *S. typhimurium* enzyme I which is significantly different from the value we have found for *E. coli* enzyme I (Hoving et al., 1981, 1982). These matters are presently under investigation.

## Acknowledgments

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**Registry No.** BrPyr, 1113-59-3; PEP, 138-08-9; Pyr, 127-17-3; Ox, 144-62-7; enzyme I, 37278-17-4; cysteine, 52-90-4.

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# Interaction of Analogues of Nicotinamide Adenine Dinucleotide Phosphate with Dihydrofolate Reductase from *Escherichia coli*†

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ABSTRACT: Steady-state kinetic techniques have been used to investigate the pyridine nucleotide specificity of dihydrofolate reductase from *Escherichia coli* as well as the binding of fragments of NADP to the free form of enzyme and to the enzyme–dihydrofolate complex. The reduced forms of nicotinamide hypoxanthine dinucleotide phosphate and acetylpyridine adenine dinucleotide phosphate function as alternative substrates but exhibit maximum velocities that are lower than with NADPH. There are only small differences in the strength of binding of the various pyridine nucleotides to the free enzyme and to the enzyme–dihydrofolate complex. The reduced form of thionicotinamide adenine dinucleotide phosphate is not a substrate, but both the reduced and oxidized forms of this nucleotide act as inhibitors. The inhibitions by these compounds involve their interaction with an enzyme–tetrahydrofolate complex. The kinetic results confirm that the dihydrofolate reductase reaction only approximates to a rapid equilibrium, random mechanism with NADPH as the pyridine nucleotide substrate. But when NADPH is replaced by reduced acetylpyridine adenine dinucleotide phosphate, both kinetic and binding data indicate that the kinetic mechanism of the reaction is truly rapid equilibrium, random. All fragments of NADP which were tested behaved as inhibitory analogues of this nucleotide. From the values of the dissociation constants obtained for their interactions with the enzyme, it is concluded that the 2′- and 5′-phosphoryl groups of adenosine make significant contributions to the binding energy of NADP whereas the nicotinamide moiety does not.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF)[1] to 5,6,7,8-tetrahydrofolate. This enzyme is the site of action of a number of imporant chemotherapeutic agents such as methotrexate, trimethoprim, and pyrimethamine. As a consequence of the clinical importance of the interaction between dihydrofolate reductase and a number of drugs, the enzyme has been studied extensively (Hitchings & Smith, 1980; Gready, 1980; Roth & Cheng, 1982). More recently, a number of investigations have been directed toward the determination of the groups involved with the binding of NADPH and its analogues to dihydrofolate reductase from various sources. Several approaches have been used, and these include X-ray crystallography (Filman et al., 1982; Matthews et al., 1978), rapid reaction techniques (Dunn et al., 1978; Dunn & King, 1980; Cayley et al., 1981), and NMR and fluroescence titration (Way et al., 1975; Birdsall et al., 1980a,b, 1981a; Feeney et al., 1980a; Gronenborn et al., 1981c; Hyde et al., 1980a,b). The majority of studies have been concerned with the formation of either binary enzyme–inhibitor or ternary enzyme–NADPH–inhibitor complexes. Much less attention has been paid to the interaction of NADPH with the enzyme–DHF complex and to groups in the pyridine nucleotide which are of importance for their binding in the ternary complex.

In the present study, steady-state kinetic techniques have been used to determine the substrate specificity of dihydrofolate reductase from *Escherichia coli* and the kinetic mechanism of the reaction with various nucleotides. In addition, determinations have been made of the strength of interaction of analogues and derivatives of NADP(H) with the free en-

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[1] Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; ADP-ribose, adenosine 5′-diphosphate ribose; ATP-ribose, 2′-phosphoadenosine 5′-diphosphate ribose; APADP, 3-acetylpyridine adenine dinucleotide phosphate; ε-NADP, nicotinamide 1,$N^6$-ethenoadenine dinucleotide phosphate; NHDP, nicotinamide hypoxanthine dinucleotide phosphate; TNADP, thionicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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