Uridine Diphosphate Galactose 4-Epimerase. Alkylation of Enzyme-Bound Diphosphopyridine Nucleotide by *p*-(Bromoacetamido)phenyl Uridyl Pyrophosphate, an Active-Site-Directed Irreversible Inhibitor†

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ABSTRACT: When UDP-galactose 4-epimerase is inactivated by *p*-(bromoacetamido)phenyl uridyl pyrophosphate (BUP), the diphosphopyridine nucleotide (DPN) associated with this enzyme as a tightly bound coenzyme cannot be reduced by substrates or by UMP-activated reduction by glucose. Upon acid denaturation of the inactivated enzyme, the DPN released corresponded to 15–30% of that released from the native enzyme. When the enzyme is inactivated by [^{14}C]BUP, about 80% of the radioactivity bound at the active site is released from the protein upon acid denaturation. When epimerase·[^{3}H]DPN is inactivated with [^{14}C]BUP, the ^{3}H and ^{14}C released from the protein upon denaturation of the complex cochromatograph on DEAE-Sephadex. Experiments with [*nicotinamide*-4-^{3}H]DPN and [*adenine*-2,8-^{3}H]DPN show that it is the adenine ring that is alkylated. The data suggest that the adenine ring of DPN in epimerase·DPN may be oriented near the glycosyl-binding subsite of this enzyme. Since the nicotinamide ring must also be near this site, it appears that the DPN may not be in an extended conformation when it is bound at the active site of UDP-galactose 4-epimerase from *Escherichia coli*.

In the preceding paper BUP[1] was shown to inactivate *Escherichia coli* UDP-galactose 4-epimerase by alkylating one or more functional groups at the active site (Wong et al., 1979). Approximately 1 mol of [^{14}C]BUP per mol of enzyme was found to be incorporated into the enzyme in this process. In subsequent experiments we found, contrary to our expectations, that most of the radioactivity incorporated into the active site from [^{14}C]BUP is not covalently bonded to a functional group of the enzyme but rather it is dissociated by acid denaturation of the protein.

In this paper we show that a major part of the alkylation at the active site involves alkylation of DPN, which is present as a tightly bound coenzyme. At least 50% of the alkylation of DPN occurs on the adenine ring, showing that this ring must be accessible to the bromoacetamido group of BUP. Inasmuch as this moiety of BUP should be in the vicinity of the glycosyl-binding subsite of the enzyme when BUP is bound, this suggests that the adenine ring of DPN^{+} is placed near this subsite.

Experimental Procedures

Paper Chromatography. The following solvents were used as the mobile phases in paper chromatographic separations: I, 95% ethanol–1 M ammonium acetate (pH 3.5), 5:2; II, 2-propanol–1% ammonium sulfate, 2:1; III, 0.1 M sodium phosphate (pH 6.8)–ammonium sulfate–1-propanol, 100:60:2; IV, 1-butanol–acetic acid–water, 120:30:50; V, solution A, 0.1 M ammonium acetate containing 100 mM EDTA adjusted to pH 9 with concentrated ammonia and saturated with sodium tetraborate, and solution B, 90% ethanol. Solutions A and B were mixed in the ratio 60:14 and allowed to stand for 60 min. The solvent was used after filtering a precipitate.

Enzymes, Coenzymes, and Chemicals. Alkaline phosphatase, pig heart lipoamide dehydrogenase, and *Crotalus adamanteus* nucleotide pyrophosphatase were obtained from Sigma Chemical Co. Other materials were obtained as described in the preceding paper (Wong et al., 1979).

Radiochemicals. [*adenine*-2,8-^{3}H]DPN was purchased from New England Nuclear and purified by paper chromatography, using solvent I as the mobile phase. The specific activity was 5.6×10^{6} cpm/μmol.

[*nicotinamide*-4-^{3}H]DPN was prepared according to eq 1 and 2 by a procedure developed in this laboratory by Pydeski (1975). The specific activity was 2.4×10^{6} cpm/μmol.

$$\text{DPN} + \text{dihydrolipoamide} \underset{\text{lipoamide dehydrogenase}}{\overset{^{3}H_2O}{\rightleftharpoons}} [\text{4-B-}^{3}\text{H}]\text{DPNH} + \text{lipoamide} \quad (1)$$

$$[\text{4-B-}^{3}\text{H}]\text{DPNH} + CH_3CHO \xrightarrow{\text{alcohol dehydrogenase}} [\text{4-}^{3}\text{H}]\text{DPN} + CH_3CH_2OH \quad (2)$$

[*bromoacetyl*-2-^{14}C]BUP was synthesized as described in the preceding paper (Wong et al., 1979).

Assays. DPN in UDP-galactose 4-epimerase was measured by reduction to DPNH or by conversion to the cyanide adduct after denaturing the protein with acid to dissociate the coenzyme. DPN^{+} was released by denaturing a 1.0-mL aliquot of enzyme with 0.12 mL of cold 35% perchloric acid. After 20 min at 0 °C the precipitate was removed by centrifugation at 6000*g* for 5 min. A 1.0-mL aliquot of the supernatant fluid was mixed with 0.12 mL of 1 M potassium Bicinate buffer at pH 8.5, and the pH was adjusted to pH 8.5 by addition of 7 M KOH (99 μL). The potassium perchlorate was removed by centrifugation, and DPN was measured in the supernatant fluid. For measurement by reduction to DPNH, a 0.9-mL aliquot was mixed with 50 μL of ethanol and 167 units of yeast alcohol dehydrogenase. The total volume of the assay mixture was 0.96 mL, and the DPN^{+} concentration was measured as DPNH by the increase in A_{340}. For measurement as the cyanide adduct, the dissociation of DPN was carried out as described above except that potassium Bicinate was replaced

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[1] Abbreviations used: BUP, *p*-(bromoacetamido)phenyl uridyl pyrophosphate; DPN, diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; NMN, nicotinamide mononucleotide; AMP, adenosine 5′-monophosphate; NR, nicotinamide riboside; Ade, adenine; Ado, adenosine; N, nicotinamide.

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with Tris-HCl buffer and the pH was adjusted to 10 with 7 M KOH (113 μL). A 0.9-mL aliquot of the resulting supernatant fluid was combined with 50 μL of 0.4 M cetyltrimethylammonium bromide and 0.2 mL of 5.5 M sodium cyanide, and the total increase in A_{327} was measured. The DPN contents of the epimerase samples used for these determinations were obtained by back-calculation from the DPN contents of the aliquots assayed.

Radiochemical assays of aqueous samples were performed by liquid scintillation counting using a scintillation solvent consisting of 7 g of 2,5-diphenyloxazole, 0.3 g of *p*-bis[2-(5-phenyloxazolyl)]benzene, and 100 g of naphthalene per L of 1,4-dioxane solution. Aqueous samples in a total volume of 1 mL were mixed with 15 mL of scintillation solvent for counting. Paper chromatograms were cut into 1-cm-wide strips (2.5–4 cm long). The strips were individually soaked overnight in 1 mL of water inside liquid scintillation vials. The following day 15 mL of scintillation solvent was added to each vial before counting. [^{14}C]Glycine was counted after dissolution in Dimilume-30 obtained from the Packard Instrument Co. All radiochemical assays were made in a Packard Tri-Carb Model 3310 liquid scintillation spectrometer. Radiochromatogram strips containing large amounts of radioactivity were scanned with a Packard Model 385 scanner.

All other assays were performed as described in the preceding paper (Wong et al., 1979).

Preparation of Epimerase·[^{3}H]DPN. [*nicotinamide*-4-^{3}H]-DPN and [*adenine*-2,8-^{3}H]DPN were exchanged with DPN endogenous to epimerase·DPN by the procedure of Davis & Glaser (1971). The exchange mixtures consisted of the following in 1.0-mL total volume: 8 mg of UDP-galactose 4-epimerase, 5 μmol of sodium *p*-(hydroxymercuri)benzoate, 100 μmol of potassium Bicinate at pH 8.5, and 0.3 μmol of [^{3}H]-DPN, either [*nicotinamide*-4-^{3}H]DPN (2.4×10^6 cpm/μmol) or [*adenine*-2,8-^{3}H]DPN (5.6×10^6 cpm/μmol). After 1 h at 27 °C, 50 μmol of β-mercaptoethanol was added to the solution. After an additional 30 min the protein was precipitated by adding 0.6 g of $(NH_4)_2SO_4$, cooling in an ice–water bath, and centrifuging. The precipitate was dissolved in 1.0 mL of 10 mM K_2HPO_4 containing 1 mM EDTA and dialyzed against several 3-L changes of the same buffer until the dialyzing buffer was free of radioactivity.

Inactivation of Epimerase·DPN by BUP and Dissociation of Products. Epimerase·DPN (3 mg/mL) was inactivated by reaction with 1.0 mM BUP at 27 °C in 50 mM potassium Bicinate buffer at pH 8.5. The reaction was monitored by assaying aliquots for residual epimerase activity at 12-h intervals. The reaction was permitted to proceed until over 90% of the activity had been destroyed, which required several days. The inactive complex was finally isolated by gel filtration through a column of Sephadex G-25 or by $(NH_4)_2SO_4$ precipitation and dialysis. The products of DPN modification resulting from the reaction were isolated from the inactive complex by the perchloric acid precipitation method described above for dissociating DPN or by precipitating the protein with trichloroacetic acid and centrifuging it. Excess trichloroacetic acid in the supernatant fluid was removed by repeated extraction with diethyl ether.

Enzymatic Degradation of BUP-Modified DPN. BUP-modified DPN obtained from inactivated enzyme was degraded to neutral products by treatment with nucleotide pyrophosphatase to cleave all pyrophosphate groups and alkaline phosphatase to remove all resulting phosphate groups. Modified DPN, labeled with both ^{3}H present initially in DPN and ^{14}C present initially in BUP, was dissociated from the inactivated complex as described above. It was degraded in the presence of 1 unit of nucleotide pyrophosphatase, 10 μmol of $MgCl_2$, 20 units of alkaline phosphatase, and 10 μmol of triethylammonium bicarbonate at a final pH of 8.5 in a total volume of 1.0 mL. The degradation proceeded at 37 °C for 4–5 h. These conditions were shown to complete the degradation of DPN to nicotinamide nucleoside and adenosine. At the conclusion of the degradation the proteins were precipitated with trichloroacetic acid, excess trichloroacetic acid was removed from the supernatant fluid by extraction several times with diethyl ether, and the aqueous layer was brought to a small volume by rotary evaporation in vacuo. Samples were spotted on paper chromatograms for chromatographic analysis beside markers of known compounds.

Results

Alkylation of DPN in Epimerase·DPN by BUP. As a preliminary evaluation of the nature of BUP-inactivated UDP-galactose 4-epimerase, we studied the reducibility of the associated DPN as compared with that in native epimerase. In the active enzyme the DPN is reduced by glucose in a UMP-dependent reaction (Bhaduri et al., 1965; Kang et al., 1975) and by millimolar concentrations of $NaBH_4$ (Nelsestuen & Kirkwood, 1971; Davis et al., 1974). UMP-dependent reducibility of DPN was examined in two reaction mixtures which differed in that one contained native epimerase and the other contained epimerase that had been more than 94% inactivated by BUP. The solutions contained, in 1.0 mL, 0.85 mg of epimerase, 1 μmol of UMP, 0.1 mmol of potassium Bicinate buffer at pH 8.5, and 0.1 mmol of glucose at 27 °C. Production of DPNH was measured as the A_{345} increase vs. that of the reference solutions in which epimerase was omitted. The reaction of the native epimerase produced 10 nmol of DPNH while the inactivated enzyme did not produce any detectable DPNH.

Native epimerase and inactivated epimerase were similarly compared with respect to reducibility by $NaBH_4$. The 0.9-mL reaction mixtures contained 0.8 mg of epimerase, 0.09 mmol of sodium carbonate buffer at pH 10, and 5 μmol of $NaBH_4$ at 27 °C. The A_{345} increases showed that 9.9 nmol of DPNH was produced in the reduction of the native enzyme and 0.97 nmol was produced in the reduction of the inactivated enzyme. This was somewhat surprising since we did not expect covalently bonded BUP to block all access to DPN by nonspecific reducing agents such as $NaBH_4$.

Native epimerase and inactivated epimerase were further compared by measuring the amounts of DPN^+ released upon acid denaturation of the proteins. Samples of native and modified proteins, 1.5 mg of each, were subjected to DPN^+ analysis by the alcohol dehydrogenase method described under Experimental Procedures. The native enzyme was found to contain 17.2 nmol of DPN while the inactivated sample contained only 5.8 nmol. When the same experiment was carried out with DPN being measured by the cyanide method, 15.5 nmol of DPN was detected in the native enzyme while only 2.4 nmol was detected in the inactivated enzyme. These results were repeatedly confirmed, and on this basis we concluded that the inactivation by BUP led either to the loss of DPN by dissociation or to the covalent modification of a major part of the DPN. That the latter is the correct explanation was shown by the fact that epimerase·[*nicotinamide*-4-^{3}H]DPN retained its radioactivity after being inactivated by BUP.

If inactivation by BUP involves alkylation of DPN, the ^{14}C bound to the active site in the course of inactivation by [^{14}C]-BUP should be readily dissociated together with DPN upon acid denaturation of the inactivated protein. In a series of five

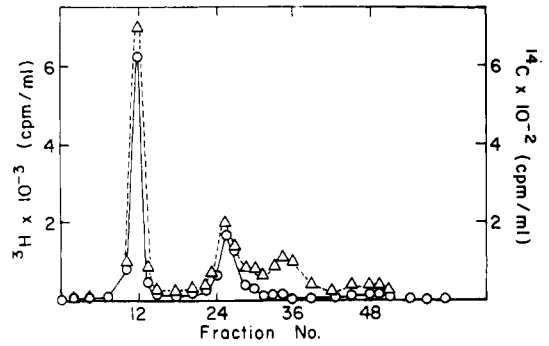


FIGURE 1: Ion-exchange chromatography of products from [^{14}C]BUP inactivation of epimerase·[*nicotinamide*-4-^{3}H]DPN. Epimerase·[*nicotinamide*-4-^{3}H]DPN$^+$ was prepared and inactivated with [^{14}C]-BUP as described under Experimental Procedures. A sample (1.4 mg) was dialyzed against 5 mM K_2HPO_4, and the DPN was dissociated by the $HClO_4$ method. After centrifugation the supernatant fluid was applied to a 1 × 20 cm column of DEAE-Sephadex A-25 which had been equilibrated with 0.1 M triethylammonium bicarbonate at pH 7.8, and the nucleotides were eluted with a 420-mL linear gradient from 0.1 to 0.75 M triethylammonium bicarbonate at 4 °C and pH 7.8. Fractions were collected at 4.5-min intervals at a flow rate of 0.67 mL/min, and aliquots of selected fractions were counted for ^{3}H and ^{14}C. Symbols: (○) ^{3}H; (△) ^{14}C.

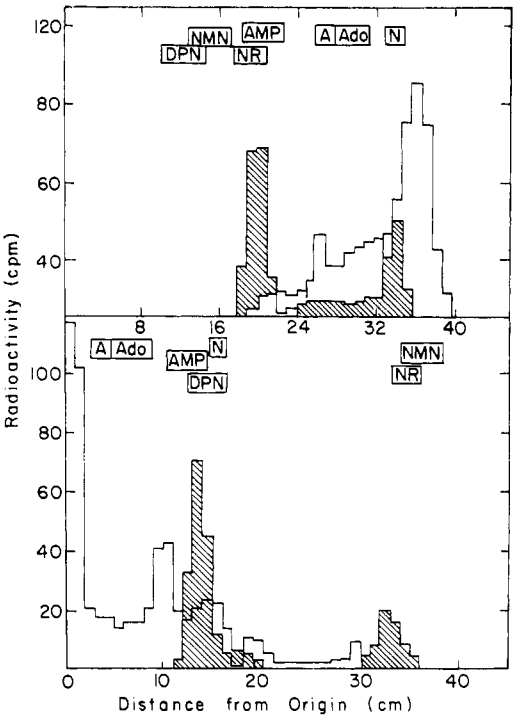


FIGURE 2: Chromatography of enzymatic degradation products of [*nicotinamide*-4-^{3}H]DPN modified by [^{14}C]BUP. Epimerase·[*nicotinamide*-4-^{3}H]DPN was inactivated with BUP and epimerase·DPN was inactivated with [^{14}C]BUP. The modified DPN was isolated from each sample and enzymatically degraded as described under Experimental Procedures. A sample containing 2220 cpm of ^{14}C was combined with one containing 1440 cpm of ^{3}H and subjected to paper chromatography by the descending technique. In the upper panel solvent system II was used and in the lower panel solvent III was used. The shaded bars represent ^{3}H and the open bars represent ^{14}C.

experiments, epimerase·DPN was inactivated with [^{14}C]BUP and isolated as described under Experimental Procedures. The total [^{14}C]BUP incorporation amounted in these experiments to 3.05 ± 0.23 mol of ^{14}C per mol of epimerase·DPN. Ultraviolet spectral analysis of native and inactivated enzyme confirmed that 3.1 mol of BUP was bound. Each sample was then subjected to denaturation with perchloric acid as described under Experimental Procedures, and the radioactivity released from the denatured protein into the supernatant fluid was measured. This amounted to 0.79 ± 0.12 mol of ^{14}C per mol of epimerase·DPN. In control experiments in which the labeling with [^{14}C]BUP was carried out in the presence of the competitive inhibitor 8-anilino-1-naphthalenesulfonate at 100 times its K_i, the incorporation of radioactivity was decreased by about 1 mol/mol of epimerase·DPN, as reported in the preceding paper (Wong et al., 1979), and none of the radioactivity incorporated was dissociated upon denaturation with acid. These results conformed with the interpretation that BUP alkylates DPN.

The fact that only about 80% of the ^{14}C bound at the active site is dissociable upon acid denaturation complements the fact that between 70 and 80% of the DPN is destroyed in the course of the inactivation, suggesting that only 70–80% of the inactivation and binding of [^{14}C]BUP involves alkylation of DPN.

More direct evidence of the alkylation of DPN by BUP was obtained in another experiment in which we inactivated epimerase·[*nicotinamide*-4-^{3}H]DPN with [^{14}C]BUP, isolated the inactive complex, dissociated the nucleotides by acid denaturation of the protein, and chromatographed the nucleotides on a DEAE-Sephadex A-25 column. As shown in Figure 1, we obtained two products that contained both ^{3}H and ^{14}C in the same ratio and a smaller amount of a third product which contained only ^{14}C. This verified the existence of a covalent linkage between DPN and BUP in the inactivated enzyme.

We do not know the exact relationship between the two ^{14}C/^{3}H products shown in Figure 1; however, they clearly differ in electrostatic charge. Inasmuch as this enzyme is known to contain DPNH rather than DPN in some of its sites (Wong et al., 1978) and the epimerase·DPNH complex is known to bind BUP with a dissociation constant of 0.027 mM (Wong & Frey, 1977), it is possible that the two products are alkylation products of DPN and DPNH, respectively, with the alkylated DPNH being present in the lesser amount and eluted second.

Localization of the Alkylated Site in DPN. To determine whether the alkylation of DPN involves the nicotinamide nucleoside portion of DPN, we inactivated epimerase·[*nicotinamide*-4-^{3}H]DPN with [^{14}C]BUP, dissociated the modified DPN from the protein, and degraded it enzymatically with nucleotide pyrophosphatase and alkaline phosphatase as described under Experimental Procedures. The degradation products were subjected to paper chromatography with two solvents, and products containing both ^{14}C and ^{3}H were sought by radiochemical analysis of the chromatograms. As shown by Figure 2, no such product could be detected. We concluded that alkylation by BUP could not have involved the nicotinamide nucleoside portion of DPN.

We next carried out the same experiment with epimerase·[*adenine*-2,8-^{3}H]DPN to determine whether the adenosine portion of DPN might contain the alkylation site or sites. We obtained the results given in Figure 3, which showed quite clearly that the adenosine portion is alkylated. A single product containing both ^{14}C and ^{3}H and migrating together in three chromatography solvents was obtained. The product migrated near but not identically with adenosine in all the solvents.

On the basis of the specificities of the degradation enzymes, this product should be an *N*-(*p*-hydroxyphenyl)carboxamidomethyl derivative of adenosine. The most likely site of alkylation in the adenosyl portion of DPN would be the purine ring at the 1 position or at the N^6-amino group.

In order to determine whether this is the case, we subjected another sample of the [*adenine*-2,8-^{3}H]DPN·[^{14}C]BUP product to mild acid hydrolysis under conditions that would cleave

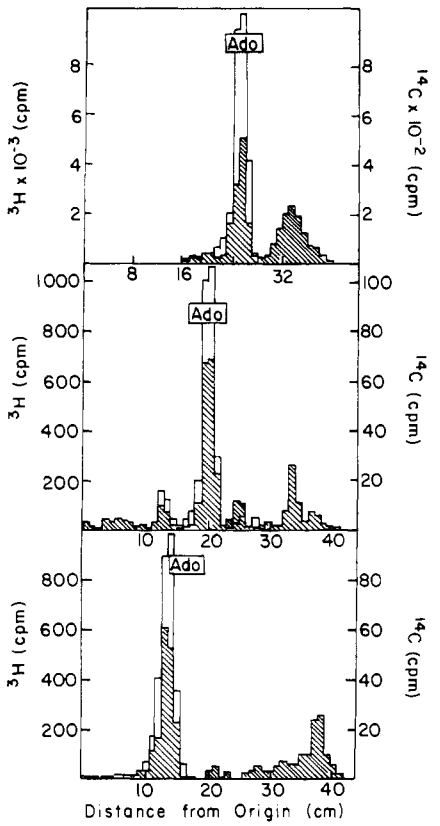


FIGURE 3: Chromatography of enzymatic degradation products of [*adenine*-2,8-^{3}H]DPN modified by [^{14}C]BUP. Epimerase·[*adenine*-2,8-^{3}H]DPN was inactivated with [^{14}C]BUP, and the modified DPN was isolated from the complex and degraded enzymatically as described under Experimental Procedures. In the upper panel a sample containing 31 500 cpm of ^{3}H and 3520 cpm of ^{14}C was subjected to descending paper chromatography using solvent system II. In the middle and lower panels the modified DPN was purified by column chromatography as described in Figure 1, and the major band eluted was subjected to enzymatic degradation as described under Experimental Procedures after volatilization of the elution buffer. Samples containing 13 170 cpm of ^{3}H and 1360 cpm of ^{14}C were subjected to descending paper chromatography using solvent IV in the center panel and solvent V in the lower panel. The shaded bars represent ^{14}C and the open bars represent ^{3}H.

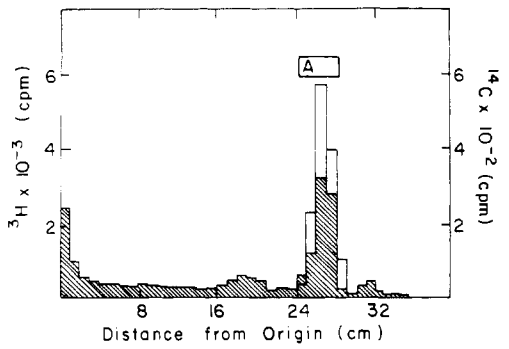
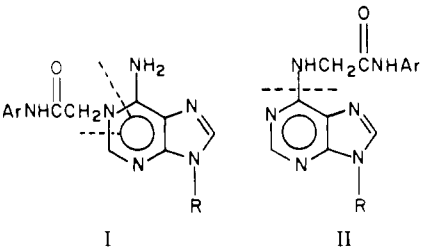


FIGURE 4: Chromatography of mild acid degradation products of [*adenine*-2,8-^{3}H]DPN modified by [^{14}C]BUP. A sample of [^{14}C]-BUP-modified [*adenine*-2,8-^{3}H]DPN was prepared as described under Experimental Procedures and hydrolyzed in 0.1 M HCl in a sealed tube at 100 °C for 2 h. HCl was removed by rotary evaporation to dryness, and the residue, which contained 5200 cpm of ^{3}H and 450 cpm of ^{14}C, was subjected to descending paper chromatography in solvent system V. The shaded bars represent ^{14}C and the open bars represent ^{3}H.

the *N*-ribosyl bond and subjected the products to paper chromatographic analysis. As shown in Figure 4, a single product containing both ^{14}C and ^{3}H was again obtained, this time migrating with adenine, although it could not be adenine, and well separated from adenosine which migrated at about half the rate in the solvent used.

It is noteworthy that substantial amounts of ^{14}C did not migrate with the single tritiated product in Figure 3 and Figure 4. A very significant amount of the ^{14}C dissociated from the enzyme is not covalently bonded to DPN, confirming the same finding in Figure 1.

Degradation of [^{14}C]*BUP-Modified DPN to* [^{14}C]*Glycine.* The foregoing experiments clearly indicate that a major alkylation site in the inactivation of epimerase·DPN by BUP is the adenine ring of DPN. The only nucleophilic nitrogen atoms in adenine are nitrogen atoms and the most probable alkylation sites are the N-1 nitrogen in the ring and the N^6-amino group. As illustrated below in structures I and II, either



I II

of these should produce glycine when exhaustively hydrolyzed in strong acid.

To determine directly whether this is the case, we inactivated epimerase·DPN with [^{14}C]BUP and isolated the modified DPN as described under Experimental Procedures. To 4360 cpm of this ^{14}C (4 nmol) which had been hydrolyzed in 6 M HCl in a sealed tube for 36 h at 120 °C was added 34.5 mg of carrier glycine. The solution was brought to dryness by rotary evaporation, and the residue was taken up in 0.3 mL of H_2O. A few drops of ethanol were added periodically at 0 °C and glycine crystals formed at this temperature. The crystals were centrifuged, washed with absolute ethanol, and recrystallized. The crystals were centrifuged, washed with ethanol, and dried. A weighed sample was counted and found to contain 4.0×10^4 cpm/g, which corresponded to 32% of the theoretical value if all of the ^{14}C had been associated with glycine. Therefore, at least 32% of the alkylation of DPN by [^{14}C]BUP occurred in the adenine ring of the coenzyme. This experiment was repeated with identical results, and the ^{14}C was shown to comigrate with glycine on paper chromatograms (1-butanol–acetic acid–H_2O, 120:30:50).

Discussion

The present work shows that the major fraction of the tightly bound inhibitor molecules is covalently bonded to the DPN$^+$ associated with the active site. This confirms that BUP inactivated the enzyme by alkylation at the active site. The result is, however, not very satisfying from the standpoint of our original intention of identifying heretofore unknown functional groups at this site.

The alkylation of DPN$^+$ by BUP, in addition to being a novel result, is of interest from the standpoint of the sites in DPN that are alkylated. The relationships between the structure of BUP and the structure of UDP-glucose or UDP-galactose are such that it is to be expected that the uridyl-pyrophosphoryl group of BUP will be bound by the enzyme at its binding site for this group in substrates. The inactivation kinetics and reversible competitive inhibition behavior of BUP are in accord with this expectation (Wong et al., 1979) which is based on the known binding properties of the enzyme (Wong & Frey, 1977; Wong et al., 1978). Therefore, the *p*-(bromoacetamido)phenyl group can be expected to be constrained in

its interactions to the general vicinity of the glycosyl-binding subsite, and this subsite should be adjacent to the nicotinamide ring of DPN in epimerase·DPN. No functional group in the nicotinamide ring is very reactive with alkylating agents, and so it is not alkylated. However, other groups on DPN are proximal enough to be alkylated. It is clear from Figures 3 and 4 that about half (51–55%) of the ^{14}C which is dissociated from the inactivated complex is bonded to the adenine ring. The only sites at which this ring can reasonably be expected to be alkylated are the N-1, N-3, and N-7 purine ring nitrogens and the 6-amino group. The fact that at least one-third of the ^{14}C released from the inactivated enzyme upon denaturation can be isolated as [^{14}C]glycine after the products are subjected to strong acid hydrolysis confirms that one or more of these nitrogen atoms are alkylated, probably N-1 and/or the 6-amino group. Since only about 60% of the ^{14}C bonded to the adenine ring could be isolated as glycine, it may be that N-3 and/or N-7 were also alkylated. These would not be quantitatively isolated as glycine in the degradation procedure.

Alkylation of the adenine ring places it in the vicinity of the *p*-(bromoacetamido)phenyl group of BUP in the epimerase·DPN·BUP complex. This group should also be near the nicotinamide ring, and this is confirmed by the fact that it totally quenches the fluorescence of DPNH in the epimerase·DPNH·BUP complex (Wong & Frey, 1977). Therefore, the adenine and nicotinamide rings of DPN appear themselves to be fairly closely placed in epimerase·DPN. It appears that DPN is bound in a conformation in which the heterocyclic rings can be placed near each other. Whether they are fully eclipsed and stacked remains to be seen, although this is not indicated by the fact that the fluorescence of DPNH in epimerase·DPNH is greatly enhanced (Wong & Frey, 1977). The fluorescence of 1,N^6-etheno-DPN in the epimerase·1,N^6-etheno-DPN complex is, on the other hand, at least 96% quenched (Pydeski, 1975), which is a property exhibited by this analogue when it is in ring-eclipsed conformations (Barrio et al., 1972). That DPN in the epimerase·DPN·BUP complex appears not to be exclusively in an extended conformation contrasts sharply with findings of extended conformations for DPN when bound at its binding sites in dehydrogenases (Rossman et al., 1975; Sloan & Mildvan, 1974; Luisi et al., 1975). The DPN may exist either in a folded conformation or in an equilibrium of more than one conformation, at least one of which involves proximal orientation of the heterocyclic rings.

About 20% of the [^{14}C]BUP bound at the active site is not released upon acid denaturation of the inactivated enzyme. Nothing is known of the group or groups to which this is bonded, and further studies on this point do not appear to be promising since it constitutes a relatively small component against a large background of nonspecifically bonded [^{14}C]-BUP.

Since only half of the dissociable ^{14}C is bonded to the adenine ring, some other group in DPN must also be alkylated at a rate that is competitive with that at the adenine ring. Figure 3 shows that this is unlikely to involve the nicotinamide riboside portion of the coenzyme. The pyrophosphoryl group and the 2′- and 3′-oxygen atoms of the ribosyl ring in the adenosyl group are the remaining possibilities. No more than 6–7% of the dissociable ^{14}C can be attributed to alkylation of the pyrophosphoryl group. This is the amount of radioactivity in strong acid hydrolysates that is found to cochromatograph with glycolic acid on silicic acid columns (Wong, 1978).

The most anomalous result from this study is the fact that, although alkylation by BUP does not involve the nicotinamide ring of DPN, the alkylation product does not appear to react with cyanide. Although rationalizations can be offered, we have no clear explanation for this.

The question of the identity of the general base responsible for deprotonating the C-4 hydroxyl group in substrates remains. Given the "openness" of the glycosyl subsite, its weak interactions with glycosyl groups, and its ability to accommodate a variety of structures (Kang et al., 1975; Wong & Frey, 1977), it is reasonable to expect the *p*-(bromoacetamido)phenyl group to alkylate the nucleophilic form of a general base in this site. On the evidence we must conclude that either the alkylating reagent cannot penetrate deeply enough into the site to reach the general base or the general base is one of the nitrogen atoms in the adenine ring of DPN. It is possible that there is some kind of steric barrier preventing the alkylation of another base; however, such a barrier would have to be presented by the enzyme itself because BUP is itself very highly effective in alkylating the cysteine in the active site of UDP-glucose dehydrogenase (Winer, 1972).[2] The resolution of this question must await the results of studies employing other active-site-directed alkylating agents.

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