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p-(Bromoacetamido)phenyl Uridyl Pyrophosphate: An Active-Site-Directed Irreversible Inhibitor for Uridine Diphosphate Galactose 4-Epimerase†

Yun-Hua Huang Wong, Fredric B. Winer, and Perry A. Frey*

ABSTRACT: The synthesis of *p*-(bromoacetamido)phenyl uridyl pyrophosphate (BUP) is described. This compound is an active-site-directed irreversible inhibitor of *Escherichia coli* UDP-galactose 4-epimerase. The inactivation follows pseudo-first-order kinetics at pH 8.5 in nonnucleophilic buffers, and a saturation effect is seen in the pseudo-first-order rate constant as the concentration of BUP is increased. The half-saturation parameter for BUP in the inactivation is 0.21 ± 0.02 mM, which compares favorably with the inhibition constant of 0.3 ± 0.05 mM for BUP acting as a competitive reversible inhibitor of the enzyme. The inactivation rate is slow, however, with a minimum half-time of 12 h at pH 8.5 and 27 °C. Both specific alkylation and nonspecific alkylation by BUP occur, but nonspecific alkylation is faster than the inactivation and the rate of inactivation correlates well with the rate of covalent incorporation of one molecule of [^{14}C]BUP at the active site.

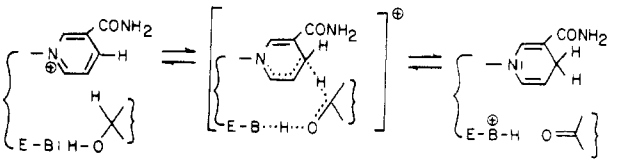
The mechanism of the interconversion of UDP-galactose and UDP-glucose catalyzed by UDP-galactose 4-epimerase is known to involve the reversible formation of an epimerase·DPNH·UDP-4-ketohexose intermediate complex, eq 1 and 2

$$E\cdot DPN^+ + \text{UDP-Gal} \rightleftharpoons E\cdot DPN^+\cdot\text{UDP-Gal} \rightleftharpoons E\cdot DPNH\cdot\text{UDP-4-ketohexose} \quad (1)$$

$$E\cdot DPNH\cdot\text{UDP-4-ketohexose} \rightleftharpoons E\cdot DPN^+\cdot\text{UDP-Glc} \rightleftharpoons E\cdot DPN^+ + \text{UDP-Glc} \quad (2)$$

(Nelsestuen & Kirkwood, 1971; Maitra & Ankel, 1971; Wee & Frey, 1973; Adair et al., 1973). The interconversions of this central complex with the epimerase·DPN$^+$·UDP-hexose complexes requires general acid–base catalysis, i.e., general base catalyzed removal of the proton from the glycosyl C-4 hydroxyl groups of UDP-hexose substrates concomitant with their conversion to the ketonic intermediate and general acid catalyzed protonation of the ketonic oxygen in concert with its reduction to the galactosyl or glucosyl groups. Since the reduction process is the microscopic reverse of the oxidation process, or nearly the microscopic reverse, the general acid and general base functions can be expected to be performed by a single functional group which acts as a general base in the oxidation of glycosyl groups and as a general acid in the reduction of the 4-ketohexopyranosyl group (Scheme I). While the identity of this group is not known, it is almost certainly present, because in the absence of such a group with a pK_a in the near-physiological range the reversible redox process would involve the compulsory formation of high-energy intermediates, either the protonated 4-ketohexopyranosyl group or the alkoxide ion.

Scheme I



A similar situation exists for all such reactions involving oxidation of an alcohol to a ketone, and general bases corresponding to that in Scheme I are known to be present in the active sites of pyridine nucleotide dependent dehydrogenases (Holbrook et al., 1975; Banaszak & Bradshaw, 1975). In these cases a histidyl residue appears to be appropriately situated to function as the prototropic catalyst.

In order to identify this functional group, we have undertaken to synthesize an active-site-directed irreversible inhibitor which may alkylate the functional group when it is in its unprotonated, nucleophilic form. In the design of the inhibitor we have taken into account the binding properties of the active site of this enzyme as well as the degradation of the alkylated enzyme to an identifiable product. These considerations led us to synthesize *p*-(bromoacetamido)phenyl uridyl pyrophosphate (BUP),[1] **1**.

† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received March 6, 1979.* Supported by Grant No. 13502 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

[1] Abbreviations used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; UMP, uridine 5′-monophosphate; Ans, 8-anilino-1-naphthalenesulfonate; NUP, *p*-nitrophenyl uridyl pyrophosphate; AUP, *p*-aminophenyl uridyl pyrophosphate; BUP, *p*-(bromoacetamido)phenyl uridyl pyrophosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

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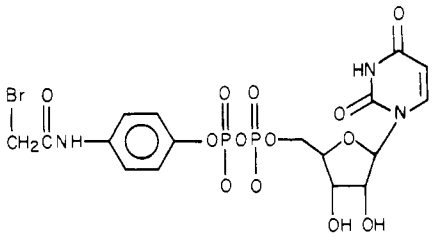

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This compound contains the uridylpyrophosphoryl group, which is known to be the major contributor to the binding free energy for both substrates and the intermediate (Kang et al., 1975; Wong & Frey, 1977, 1978), so it should have good binding affinity for the active site. The *p*-(bromoacetamido)-phenyl group is bonded to the nucleotide in place of the glycosyl group of the substrates. This substitution should not greatly affect the binding specificity or affinity because glycosyl groups of substrates, inhibitors, and intermediates contribute little to the binding free energy of uridine nucleotides at the active site (Wong & Frey, 1977). The *p*-(bromoacetamido)phenyl group is a reasonably effective alkylating group introduced by Wilchek & Anfinsen (1969) in their chemical mapping studies of the active site of staphylococcal nuclease. It is a convenient group for such work because upon complete hydrolysis of an alkylated protein the alkylated α-amino acid should be a carboxymethyl derivative, which should be readily identified.

In this paper we report on the synthesis of BUP, on the inactivation of UDP-galactose 4-epimerase by this compound, and on the stoichiometry of [^{14}C]BUP incorporation. BUP is here shown to be an active-site-directed alkylating agent for this enzyme, and in an accompanying article it is shown to alkylate the adenine ring of the DPN molecule that is present as a tightly bound coenzyme at the active site of this enzyme (Wong & Frey, 1979).

Experimental Procedures

Materials. UDP-galactose 4-epimerase was purified from a regulatory mutant of *Escherichia coli* K12 (ATCC 27797) by a slight modification of the procedure of Wilson & Hogness (1964). Galactose-1-phosphate uridylyltransferase was purified from the same organism by Dr. Sue-Lein Lee Yang in this laboratory and kindly donated for this study. UDP-glucose dehydrogenase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, nucleotide pyrophosphatase, and alkaline phosphatase were obtained from Sigma Chemical Co. UMP, TPN$^+$, glucose 1,6-diphosphate, glucose 1-phosphate, galactose 1-phosphate, UDP-glucose, UDP-galactose, and DPN$^+$ were also obtained from Sigma. Other chemicals were obtained commercially and used without further purification except for Ans and pyridine. Ans was purchased from Sigma and twice recrystallized from water. Pyridine was redistilled and stored over KOH pellets.

Assays. The concentration of UDP-galactose 4-epimerase used in the binding experiments was measured spectrophotometrically at 345 and 280 nm. The best measure of the content of active enzyme in any preparation of this enzyme has been found to be the content of DPN$^+$ that is reducible by glucose in the presence of UMP (Kang et al., 1975). The increase in A_{345} due to DPNH formation in each enzyme preparation used in this work upon adding glucose and UMP was used to calculate the active enzyme concentration, assuming an extinction coefficient of 6.2×10^3. This was then correlated with the A_{280} of the enzyme and with the color development in the Lowry assay for protein. The concentration of inactivated enzyme was measured by the Lowry method (Lowry et al., 1951).

The assay method for UDP-galactose 4-epimerase was that of Wilson & Hogness (1964) except in the experiments to evaluate BUP as a competitive reversible inhibitor. A different assay method was used for this purpose. The amount of UDP-glucose produced from UDP-galactose in a timed incubation was measured as TPNH after adding galactose-1-phosphate uridylyltransferase, galactose 1-phosphate, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and TPN$^+$. The reaction mixture contained initially, in 0.05 mL, 12.5 nmol of UDP-galactose, 0.125 μmol of potassium Bicinate buffer at pH 8.5, BUP at various concentrations, and less than 0.2 unit of enzyme. After 5 min at 27 °C the reaction was terminated by heating at 100 °C for 3 min. After cooling, we mixed the solution with 0.5 mL of another solution which contained 1 μmol of galactose 1-phosphate, 16 μmol of cysteine, 1.3 μmol of TPN$^+$, 4 nmol of glucose 1,6-diphosphate, 5 μmol of $MgCl_2$, 5 μmol of NaF, 114 μmol of sodium Bicinate at pH 8.5, 0.2 unit of glucose-6-phosphate dehydrogenase, 0.4 unit of phosphoglucomutase, and less than 0.01 unit of galactose-1-phosphate uridylyltransferase (Wong & Frey, 1974). After 2 h at 27 °C the A_{340} was measured vs. a control solution to which no UDP-galactose 4-epimerase had been added. The unit of activity was the standard unit defined by Wilson & Hogness (1964).

Radioactivity was measured by liquid scintillation counting with a Packard Tri-Carb Model 3310 spectrometer. The scintillation medium contained 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. A 1.0-mL aqueous sample was mixed with 15 mL of this scintillator.

Synthesis of BUP. Disodium *p*-nitrophenyl phosphate (4 mmol) was dissolved in 5 mL of H_2O and converted to the pyridinium salt by passage through a 2.5 × 26 cm column of Dowex 50W-X12 cation-exchange resin in the pyridinium form. The salt was reduced to dryness by rotary evaporation in vacuo (bath temperature 30 °C). The residue was again evaporated several times after addition of 10-mL portions of anhydrous pyridine to remove the last traces of water. The residue was dissolved with 4 mmol of trioctylamine and 20 mL of anhydrous pyridine, evaporated to dryness, and dissolved in 20 mL of anhydrous pyridine containing 1.32 mmol of UMP-morpholidate. After evaporation 3 times with additions of anhydrous pyridine, the residual oil was dissolved in 5 mL of dry pyridine and placed at 45 °C for 2 days. The solvent was removed by rotary evaporation, and the residue was stirred with a small amount of water containing 600 mg of lithium acetate. This mixture was extracted several times with diethyl ether, and the aqueous layer was adjusted with HCl to pH 3.5. This solution was applied to a 4 × 40 cm column of DEAE-cellulose in the Cl^- form which had been equilibrated with 1 mM HCl. The column was eluted with a linear gradient consisting of 1 L of 0.04 M LiCl in 1 mM HCl and 1 L of 1.5 M LiCl in 1 mM HCl at a flow rate of 1 mL/min. Fractions were collected and monitored spectrophotometrically at 260 nm. Two minor bands of A_{260} were eluted ahead of the major band. The first was pyridine and the second, eluted at the midpoint of the gradient, contained *p*-nitrophenyl phosphate and UMP. The major band eluted in the last 25% of the gradient was NUP. These fractions were pooled, adjusted to pH 5 with LiOH, and evaporated to dryness. The residue was dissolved in a minimal volume of cold methanol and precipitated by addition of 10 volumes of diethyl ether. The precipitate was collected by centrifugation, and the dis-

solution with methanol and precipitation with ether were repeated until the supernatant fluid was free of Cl^-. Li_2NUP was obtained in a yield of 507–598 mg (73–84%). The uracil/phosphate/*p*-nitrophenol ratio was found to be 1.0:2.1:1.0 upon degradation with nucleotide pyrophosphatase and alkaline phosphatase at pH 8, followed by spectrophotometric analysis at 260 nm for uracil and at 400 nm for *p*-nitrophenol and colorimetric analysis for phosphate. Pure Li_2NUP exhibited an extinction coefficient at pH 7.5 of 11×10^3 at its absorption maximum of 268 nm.

NUP was hydrogenated to AUP in 1.5 h at 32 psi and room temperature in a Parr hydrogenation apparatus. The hydrogenation mixture consisted of 100 mg of NUP dissolved in 100 mL of 50% aqueous methanol (v/v) and mixed with 32 mg of 10% Pd on charcoal catalyst. After filtration of the catalyst, the solution was evaporated to dryness. The residue was dissolved in a minimal volume of methanol and precipitated with ether. The precipitate was dissolved in a minimum of methanol and again precipitated with ether. AUP obtained in over 90% yield was homogeneous upon paper chromatography and exhibited an extinction coefficient of 8.3×10^3 at pH 7.5 at its absorption maximum of 262 nm.

N-Hydroxysuccinimide bromoacetate and *N*-hydroxysuccinimide [2-^{14}C]bromoacetate, prepared according to Santi & Cunnion (1974), were used to bromoacetylate AUP to BUP. AUP (150 mg, 0.3 mmol) was dissolved in 1.5 mL of water and combined with 4.2 mL of redistilled dioxane. This was combined with 177 mg (0.75 mmol) of *N*-hydroxysuccinimide bromoacetate, and the solution was gently stirred for 3–4 h at room temperature. One volume of water was added, and the resulting solution was extracted several times with equal volumes of ether. The aqueous layer was adjusted with HCl to pH 3.5 and applied to a 1.5 × 42 cm column of DEAE-Sephadex A-25 in the Br^- form. The column was eluted with a linear gradient consisting of 250 mL of 0.05 M LiBr in 1 mM HCl and 250 mL of 0.35 M LiBr in 1 mM HCl. Fractions of 4.0-mL volume were collected at 6-min intervals at 4 °C. Two very minor bands were eluted near the midpoint of the gradient, and BUP was eluted as the major band in the last 25% of the gradient. The fractions containing BUP were pooled, adjusted to pH 5 with LiOH, and evaporated to dryness. The residue was dissolved in methanol, and Li_2BUP was precipitated by addition of diethyl ether, as described for NUP. Analytically pure dilithium BUP was obtained in 50% yield. Anal. Calcd: C, 29.93; H, 3.54; N, 6.16; Br, 11.7. Found: C, 29.69; H, 3.64; N, 6.05; Br, 11.41. The compound exhibited an extinction coefficient of 1.6×10^4 at pH 7.0 at its absorption maximum of 258 nm.

[*bromoacetyl*-2-^{14}C]BUP was prepared by an otherwise identical but scaled-down procedure. Sodium [2-^{14}C]bromoacetate was obtained from Amersham/Searle. The specific activity of the [^{14}C]BUP was 1×10^6 cpm/μmol.

Results and Discussion

Synthesis of BUP. The synthetic procedure we have developed for preparing BUP begins with the reaction of *p*-nitrophenyl phosphate with the trioctylammonium salt of UMP-morpholidate in pyridine to produce NUP. This reaction is carried out by the procedure introduced by Moffatt & Khorana (1958) which is in general use for the synthesis of nucleotide sugars (Moffatt, 1966). NUP is reduced to AUP by catalytic hydrogenation of the nitro group, and AUP is bromoacetylated to BUP by reaction with bromoacetyl-*N*-hydroxysuccinimide. The latter two steps are adapted from the analogous reactions used by Wilchek & Anfinsen (1969) to synthesize *p*-(bromoacetamido)phenyl nucleotide analogues of staphylococcal nuclease substrates. BUP is obtained analytically pure by the procedure described under Experimental Procedures.

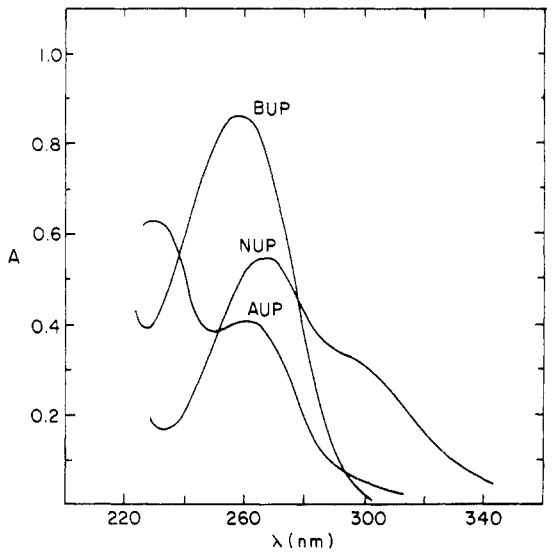

FIGURE 1: Ultraviolet spectra of BUP and synthetic intermediates. The ultraviolet spectra of 0.055 mM BUP in 0.1 M KP_i buffer at pH 7.0 and of 0.050 mM NUP and AUP in 0.1 M KP_i buffer at pH 7.5 are shown.

The ultraviolet spectra of NUP, AUP, and BUP are given in Figure 1 in support of the structure assigned to BUP.

The structure of NUP is defined by the synthetic procedure. The only functional group that can be hydrogenated under the conditions used is the nitro group, since hydrogenation of uracil requires high pressure and a nickel catalyst, so the structure of AUP is unambiguous. The ultraviolet spectrum of NUP in Figure 1 is approximately the sum of the spectral components, whereas AUP is clearly hypochromic with an extinction coefficient at 260 nm that is smaller than that of UMP itself. It appears that the delocalization of the electron pair on the amino group enriches the electron density in the phenyl ring enough to promote a stacking interaction with the uracil ring. Upon bromoacetylation, hypochromicity is relieved in BUP, presumably because the electron pair on the amide nitrogen is now less delocalized into the phenyl ring because of its delocalization into the bromoacetyl group. This suggests that it is the anilino nitrogen that is bromoacetylated.

Inactivation of UDP-Galactose 4-Epimerase by BUP. An active-site-directed irreversible inhibitor inactivates its target enzyme by forming a specific association complex at the active site and then chemically modifying one or more functional groups of the protein at this site, usually by forming a covalent bond to the group. The kinetics for such inactivation is known by the work of Fahrney & Gold (1963) on inactivators of α-chymotrypsin to be described by eq 3 and 4. In eq 4, k_{obsd}

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E^* \qquad (3)$$

$$k_{obsd} = \frac{k_3[I]}{K + [I]} \qquad (4)$$

is the pseudo-first-order rate constant for inactivation of the enzyme E by a substantial excess of the inhibitor I. The saturation parameter K is $(k_2 + k_3)/k_1$ when the appropriate approximation is the steady-state approximation. When $k_3 \ll k_2$, equilibrium binding is the appropriate approximation; K is then k_2/k_1 (Kitz & Wilson, 1962).

Inactivation of epimerase is in accord with this model, as shown by Figure 2 which gives representative inactivation data.

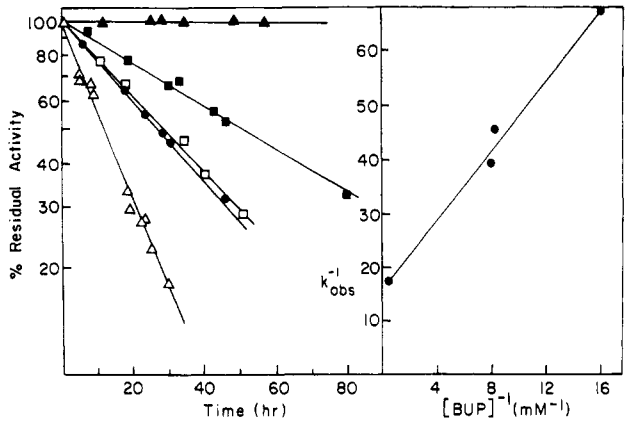

FIGURE 2: Kinetics for inactivation of epimerase by BUP. Left panel: residual activity of UDP-galactose 4-epimerase was measured in the course of inactivation by BUP in reaction mixtures which consisted of 0–2.27 mM BUP, 0.3–0.9 mg/mL UDP-galactose 4-epimerase, and 0.1 M sodium Bicinate buffer at pH 8.5 and 27 °C. The percent of residual activity was plotted vs. time on semilog graph paper, and k_{obsd} values were calculated from the slopes of the plots. Symbols: (▲) no BUP; (■) 0.062 mM BUP; (□) 0.12 mM BUP; (●) 0.125 mM BUP; (△) 2.27 mM BUP. Right panel: reciprocals of the k_{obsd} values calculated from the slopes of the first-order plots are plotted vs. the reciprocals of the corresponding BUP concentrations.

The linearity of first-order plots for 80–90% of the reaction verifies that the reagent is adequately stable under the conditions of Figure 2. At higher pH values the first-order plots curve upward (Winer, 1972), which is indicative of the decomposition of BUP. The maximum inactivation rate constant under the conditions of Figure 2 is 0.059 h^{-1}, which corresponds to a half-time of 12 h. The fact that inactivation is very slow suggests that the saturation parameter K given in Table I is the dissociation constant for BUP.

Active-Site Specificity of BUP. Although Figure 2 clearly shows that BUP inactivates epimerase according to the expected rate law, it does not by itself constitute convincing evidence that inactivation involves the interaction of BUP at the active site. It is possible that BUP may function as a nonspecific alkylating agent which inactivates the enzyme by alkylating one or more groups outside the active site which are essentially involved in maintaining the structural integrity of the enzyme. Alkylation of such groups could interfere with their structure-maintaining roles and thereby cause the enzyme to undergo a structural transition to an inactive conformation.

The establishment of active-site specificity is a general requirement for evaluating the efficacy of active-site-directed irreversible inhibitors. In the present case the fact that the inactivation is so slow makes it especially important to evaluate specificity in a critical way. The following lines of evidence are presented in support of our contention that the inactivation of epimerase by BUP results from an alkylation reaction at the active site.

If inactivation by BUP is the result of nonspecific alkylation, the same inactivation should occur in the presence of all other chemically similar alkylating reagents. However, the activity of this enzyme is completely stable for 5 days in the presence of 0.1 mM bromoacetate or bromoacetamide (Winer, 1972). This is inconsistent with the interpretation that inactivation results from the action of BUP as a nonspecific alkylating reagent.

Since irreversible inactivation by BUP is very slow, undetectable during the 2 or 3 min required for an activity assay, it should be possible to determine whether BUP is a competitive reversible inhibitor. This will be the case if BUP is reversibly bound at the active site, and the inhibition constant should be the dissociation constant, i.e., the same as the saturation parameter K obtained in the kinetics for irreversible inactivation.

Table I: Dissociation Constants for Nucleotides Binding to Epimerase

nucleotide	dissociation constant (mM)[a]	method or ref
BUP	0.21 ± 0.02	inactivn kinetics
BUP	0.30 ± 0.05	competitive inhibn
UMP	1.5	inhibn of inactivn
UMP	1.86 ± 0.5	Kang et al. (1975)
UMP	1.2	Wong & Frey (1977)

[a] All determinations were carried out at pH 8.5 and 27 °C in 0.1 M sodium Bicinate buffer.

The standard activity assay for UDP-galactose 4-epimerase (Wilson & Hogness, 1964) could not be used for evaluating BUP as a reversible inhibitor because this method involves the coupling of UDP-glucose formation to the reduction of DPN^+ to DPNH catalyzed by UDP-glucose dehydrogenase, and BUP is an exceedingly potent inactivator of this dehydrogenase. Therefore, an alternative assay method, dscribed under Experimental Procedures, was devised and used for this purpose. Since galactose-1-phosphate uridylyltransferase is not inactivated by BUP, this was used to convert UDP-glucose produced by UDP-galactose 4-epimerase to glucose 1-phosphate, which was then measured as TPNH in the presence of glucose-6-phosphate dehydrogenase and TPN^+. By use of this assay method, BUP was found to be a good competitive inhibitor of epimerase, with an inhibition constant of 0.3 ± 0.05 mM. If the mechanism of inactivation by BUP involves preequilibrium binding at the active site, reversible competitive inhibitors of UDP-galactose 4-epimerase such as UMP inhibit inactivation by competing with BUP for binding at the active site. Thus, UMP acting as a competitive reversible inhibitor of inactivation by BUP should not affect the maximum inactivation rate constant but should increase the apparent dissociation constant for BUP. The inhibition constant for UMP, measured in such experiments, should be the same as its inhibition constant as a competitive reversible inhibitor of the enzyme. UMP does indeed act as a competitive reversible inhibitor of inactivation by BUP and the inhibition constant is 1.5 mM which, as shown in Table I, is closely comparable with the dissociation constant as measured by two other techniques, including competitive inhibition of the catalytic activity of the enzyme.

The available data on the dissociation constants for BUP and UMP are assembled in Table I. From these it can be concluded that BUP competes with both UDP-galactose and UMP for binding to the enzyme. Moreover, since UMP binds at only one site in the millimolar concentration range (Wong & Frey, 1978), it is highly probable that BUP does too. The dissociation constants in Table I strongly imply that BUP acts by binding at the active site.

For the determination of how much BUP is covalently bonded to the enzyme in the course of inactivation, a number of experiments were performed with [^{14}C]BUP. The reactions were carried out at 27 °C in 0.1 M sodium Bicinate buffer at pH 8.5 with 1 mM [^{14}C]BUP (1×10^6 cpm/μmol) until the residual activity of the enzyme reached less than 5% of native activity. The protein was then isolated from unreacted [^{14}C]BUP by gel filtration and counted. In five experiments the total [^{14}C]BUP incorporated amounted to 3.05 ± 0.3 mol/mol of epimerase, which clearly showed that alkylation had occurred at sites other than the active site. This was

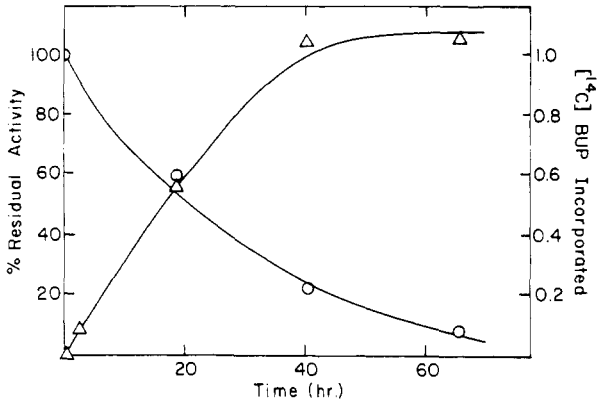

FIGURE 3: Correlation between activity loss of epimerase and incorporation of [^{14}C]BUP at the active site. The method by which the competitive inhibitor Ans was used to block [^{14}C]BUP incorporation at the active site in order to determine the extent of incorporation is described in the text. The reaction mixtures consisted of 3 mg/mL UDP-galactose 4-epimerase and 1 mM [^{14}C]BUP in 0.1 M potassium Bicinate buffer at pH 8.5. One of the two solutions also contained 2.5 mM Ans, which was 100 times its dissociation constant. Aliquots were removed at time intervals and assayed for residual activity, which is plotted for that solution which contained no Ans. Ans protected the other solution from loss of activity. At the same time intervals 0.2-mL aliquots were removed from each solution and passed through 1.5 × 30 cm columns of Sephadex G-25 equilibrated and eluted with 10 mM K_2HPO_4. The fractions containing protein were analyzed for protein and ^{14}C content, and the differences in the number of moles of ^{14}C per mole of protein in the two solutions were plotted as [^{14}C]-BUP incorporated. Symbols: (○) residual activity; (△) [^{14}C]BUP incorporated.

unsurprising because of the fact that the inactivation is so slow that nonspecific alkylation could be expected to occur in *addition* to the specific reaction at the active site. The time course for alkylation was faster than that for inactivation, indicating that active-site-directed alkylation was slower than nonspecific alkylation of the other sites. When these experiments were performed with UMP present together with [^{14}C]BUP, the degree of labeling was decreased but it could not be abolished by the presence of UMP. This suggested that labeling at the active site could be selectively repressed by the association of UMP at this site. It proved to be impractical, however, to obtain very clear data showing that UMP blocks alkylation at only one site because an acceptable inactivation rate required the use of about 1 mM [^{14}C]BUP, which is 5 times the dissociation constant for BUP, while the dissociation constant for UMP, 1.5 mM, was such that 7.5 mM would be required just to inhibit the inactivation rate by 50%. Reduction of the inactivation rate by 90% would have required the use of UMP at 81 mM, or about 30 mg/mL. Therefore, we chose to use for this purpose another, much more potent competitive inhibitor of the enzyme, Ans, which binds specifically at the active site with a dissociation constant of 25 μM (Wong & Frey, 1978).

Two alkylation experiments with [^{14}C]BUP were performed, one in the presence and one in the absence of a sufficiently large [Ans] to block alkylation at the active site. The total incorporation of ^{14}C into the enzyme was measured at selected times in both solutions, and the difference was taken as representing the number of moles incorporated at the active site.

The data are set forth in Figure 3. Note that Ans blocks both inactivation and the incorporation of 1 mol of ^{14}C per mol of enzyme. Note further that the progress curves for inactivation and alkylation at the site protected by Ans intersect at the common half-time of 20 h. This represents a close correlation between alkylation at the active site and inactivation of the enzyme.

We conclude that BUP is an active-site-directed irreversible inhibitor for *E. coli* UDP-galactose 4-epimerase. Although alkylation occurs at sites other than the active site at faster rates than the rate of alkylation at this site, there can be little doubt that alkylation does occur at the active site, that this can be specifically repressed by the presence of competitive inhibitors, and that alkylation at this site leads to the covalent bonding of 1 mol of the affinity labeling agent per mol of enzyme concomitant with inactivation.

The very slow rate of reaction at the active site is of some concern, and this is dealt with in the following paper (Wong & Frey, 1979) in which it is shown that BUP alkylates the enzyme-bound DPN.

In marked contrast to its sluggish reaction with epimerase, BUP is an exceedingly potent and specific inactivator of UDP-glucose dehydrogenase (Winer, 1972).[2] This type of reagent should be useful for alkylating nucleophilic groups in the active sites of a broad range of enzymes which catalyze reactions of the sugar moieties of nucleotide sugars. The enzymes of deoxy sugar formation should be susceptible to inactivation and derivatization by such reagents in which the uridyl group is replaced by the thymidyl or cytidyl groups.

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