reduced by the presence of extradiskal ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). This can probably be attributed to depletion of the intradiskal calcium as a result of the low extradiskal calcium concentration. Similar considerations must be taken into account for experiments which attempt to elucidate the influence of other calcium binding substances such as ATP and GTP on disk membrane properties.

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Registry No. Ca, 7440-70-2; La, 7439-91-0.

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Nucleoside and Nucleotide Inactivation of R17 Coat Protein: Evidence for a Transient Covalent RNA–Protein Bond†

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ABSTRACT: R17 coat protein forms a specific complex with a 21-nucleotide RNA hairpin containing the initiation site for the phage replicase gene. The RNA binding activity of the protein is inhibited by prior incubation with 5-bromouridine (BrU). The inactivation occurs with pseudo-first-order kinetics, and the inactive protein is stable to dilution. RNA binding activity of the BrU-inactivated protein is restored upon incubation with dithiothreitol. Inactivation of coat protein by *N*-ethylmaleimide or *p*-(chloromercuri)-benzenesulfonate indicates that a cysteine residue is located near the RNA binding site. Since 5-bromodeoxyuridine does not inactivate coat protein, a specific binding event appears to be required before inactivation can occur. Surprisingly, unmodified cytidine nucleotides also inactivate coat protein, with a specificity similar to the modified analogues. These results are discussed with regard to the formation of a transient covalent RNA–protein bond.

The translational repression of bacteriophage R17 replicase gene expression by the phage coat protein has proven to be an excellent system for the detailed study of a specific RNA–protein interaction (Uhlenbeck et al., 1983). The coat protein binds specifically to a single RNA hairpin in the initiation region of the replicase gene, thereby preventing initiation of translation (Bernardi & Spahr, 1972). A synthetic 21-nucleotide-long RNA fragment corresponding to the binding site was found to have the same affinity as R17 RNA for the binding of coat protein (Krug et al., 1982; Carey et al., 1983a). Subsequent experiments have shown that substitution of one

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of the uridines in the single-stranded portion of the 21-mer has a curious effect on coat protein binding (Carey et al., 1983b). Replacement of this uridine by either an adenosine or a guanosine results in a decreased binding affinity to coat protein, while replacement by cytidine results in an approximate 90-fold increase in the association constant (P. T. Lowary, unpublished experiments).

A special role has been proposed for the constant uridine at position 8 of tRNA molecules in their interaction with their cognate tRNA synthetases (Starzyk et al., 1982). Schoemaker & Schimmel (1977) have shown that aminoacyl-tRNA synthetases catalyze the exchange of tritium from water to the 5-carbon of uridine-8 of their cognate tRNAs. The mechanism of this exchange is believed to involve the attack of an enzyme nucleophile upon the 6-carbon of uridine, resulting in a reactive carbanion at the 5-position which quickly protonates. Abstraction of a proton from the 5-carbon of the subsequent dihydro intermediate results in full reversibility. Thymidylate synthetase uses a similar mechanism to activate the 5-carbon of dUMP for reaction with tetrahydrofolate (Santi et al., 1978). Cysteine is the most likely candidate for the nucleophile in this mechanism (Wataya et al., 1980), and both enzymes are sensitive to sulfhydryl-modifying reagents. A variety of 5-substituted pyrimidines have been useful substrate analogues in studying the mechanism of both enzymes. Both 5-azauridine and several 5-halogenated uridines will successfully inactivate thymidylate synthetase by trapping the Michael adduct (Santi et al., 1978). At high concentrations of 5-bromouridine and in the absence of sulfhydryl reagents, tRNA synthetases are also inactivated by the nucleotide through an unknown mechanism (Starzyk et al., 1982).

Since R17 coat protein has two uncomplexed cysteines (Weber, 1967) and substitution of a U by C in the RNA increases affinity for the protein, we considered the possibility that a transient covalent RNA–protein adduct forms in the translational repression complex. The results reported here demonstrate that a sulfhydryl group on R17 coat protein is essential for RNA binding and that a variety of 5-substituted pyrimidines inactivate coat protein in a manner similar to the tRNA synthetases. Surprisingly, even certain cytidine nucleotides can also inactivate coat protein.

Experimental Procedures

Materials. 5-Bromouridine (BrU) was purchased from Aldrich and Sigma. We have observed that some samples of BrU lose their ability to inactivate coat protein upon prolonged storage. Repurification by reverse-phase high-pressure liquid chromatography (HPLC) restores the activity of the BrU. Cytidine 3′,5′-bisphosphate was purchased from P-L Biochemicals. The 2′(3′),5′-bisphosphates or BrU and 3-deazauridine were synthesized according to Barrio et al. (1978) and were the kind gift of W. Wittenberg. All other nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO).

R17 coat protein was purified from phage as described previously (Carey et al., 1983a). The 21-nucleotide binding fragment was synthesized with an internal ^{32}P label (Krug et al., 1982).

Inactivation of Coat Protein. Coat protein was incubated with each nucleoside at room temperature (23 ± 1 °C) in TMK buffer [100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.5, 80 mM KCl, and 10 mM $MgCl_2$]. After the indicated incubation time, each modified protein was assayed for RNA binding in the following way. Each protein sample was serially diluted with cold TMK buffer to yield a set of concentrations ranging from 0.1 nM to 1 μM. After 5–15 min at 2 °C, ^{32}P-labeled 21-mer (final concentration 10 pM) was added to each tube, and incubation was continued for an additional 20 min. An aliquot of each reaction was then filtered on a nitrocellulose filter. After being dried, each filter was counted to generate a binding curve (Carey et al., 1983a). The coat protein concentration required to reach half-plateau binding of the RNA is equal to the dissociation constant (K_d) of the complex. In control experiments, coat protein was incubated at room temperature in TMK buffer in the absence of added nucleoside. The fractional RNA binding activity is expressed as the ratio of the apparent K_d measured with protein incubated with nucleoside to the K_d measured with protein incubated in the absence of added nucleosides.

In experiments to demonstrate the reversal of coat protein–nucleotide complexes by dithiothreitol (DTT), coat protein (16 μM) was incubated the indicated time with 80 mM nucleoside in TMK buffer at 23 °C. After an aliquot was removed to assay for RNA binding activity, the incubation mixture was diluted 40-fold with TMK buffer with or without DTT. Aliquots for the reaction were removed at the indicated time and assayed for RNA binding activity as described above.

The inactivation of coat protein with *N*-ethylmaleimide (Sigma) or *p*-(chloromercuri)benzenesulfonate (Sigma) was carried out by incubating 4 μM coat protein with different concentrations of reagent in TMK buffer at room temperature for 1 h. The inactivated proteins were assayed for RNA binding activity as described above.

Modification of BrU by Coat Protein. BrU (8 mM) was incubated for 18 h in the presence and absence of 4 μM R17 coat protein at 23 °C in TMK buffer at pH values between 7.0 and 9.0. Reactions were repeated with 10 mM DTT. The products of these reactions were analyzed by reversed-phase HPLC using a 0.46 × 25 cm ODS-Hypersil C-18 column eluted with a linear gradient from 100% buffer A to 70% buffer A/30% buffer B in 20 min. Buffer A is 50 mM ammonium acetate, pH 6.5, and buffer B is 50 mM ammonium acetate, pH 6.5, containing 70% (v/v) methanol. Under these chromatographic conditions, uridine elutes at 10.4 min, 5-bromouracil (BrUra) elutes at 12.1 min, and 5-bromouridine elutes at 16.8 min.

Results

Inactivation of R17 Coat Protein by 5-Halopyrimidines. When 14 μM R17 coat protein is incubated for 24 h with 80 mM 5-bromouridine in TMK buffer, its capacity to bind 21-mer is substantially reduced (Figure 1). This inactivation cannot result from direct competition between 5-bromouridine and 21-mer for the coat protein since incubation is required for inactivation, and once formed, the inactive complex is stable to dilution and further incubation (Figure 1). The nucleoside-treated protein has an estimated K_d for the 21-mer of 2 μM while the untreated control has a K_d of 10 nM. Since the protein is an excess of the RNA in these experiments, the 50-fold increase in K_d corresponds to a 98% inactivation of the coat protein.

The rate of inactivation of coat protein by 80 mM 5-bromouridine follows first-order kinetics (Table I). Surprisingly, inactivation appears to be considerably slower at 14 μM protein than at 4 or 0.4 μM. This result is probably related to the aggregation properties of the coat protein. In TMK buffer, R17 coat protein is a dimer within the concentration range of 10 nM to 1 μM, but above 10 μM, a substantial proportion of the protein forms capsids (D. Beckett, unpublished observations). Since the capsids have reduced RNA binding activity (Carey et al., 1983a), it is not surprising that

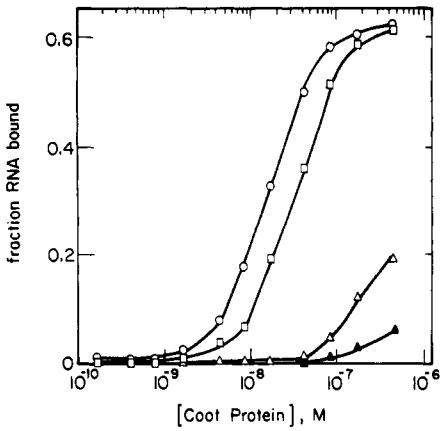


FIGURE 1: Assay of 21-mer binding to BrU-treated R17 coat proteins. R17 coat protein (14 μM) was incubated for 24 h in the presence (△) or absence (○) of 80 mM BrU. The inactivated protein was diluted 40-fold in the presence (□) or absence (▲) of 10 mM DTT and incubated an additional 12 h.

Table I: Kinetics of Inactivation of R17 Coat Protein[a]

inactivator	coat protein (μM)	K^b ($\times 10^3$ min^{-1})
BrU	0.4	20 ± 2
BrU	4.0	15 ± 2
BrU	14.0	1.3 ± 0.2
3′-CMP	4.0	1.8 ± 0.2

[a] R17 coat protein was incubated with 80 mM inactivator and assayed for RNA binding activity at several times. [b] The rate constant of inactivation is determined from the slope of the linear plot of ln (fraction of activity) vs. time.

Table II: Effect of Nucleoside Concentration on Inactivation of R17 Coat Protein[a]

inactivator	concn (mM)	fractional act.
BrU	0	1.0
	10	0.50
	20	0.46
	30	0.42
	40	0.21
	60	0.12
	80	0.02
	160	0.016
3′-CMP	0	1.0
	20	0.85
	40	0.71
	80	0.58
	120	0.40
	160	0.24

[a] R17 coat protein (4 μM) was incubated for 13 h with the indicated concentrations of inactivator and then assayed for RNA binding activity.

they also are inactivated more slowly. At a fixed incubation time, the extent of inactivation of coat protein is directly proportional to the 5-bromouridine concentration (Table II). No indication of saturation is observed up to concentrations of 5-bromouridine of 160 mM.

Both the rate and concentration dependence of inactivation of coat protein by 5-bromouridine closely resemble inactivation data for tRNA synthetases. For example, at 80 mM 5-bromouridine, 1 μM alanine–tRNA synthetase has a half-time of inactivation of 41 min (Starzyk et al., 1982) while 0.4 μM coat protein has a half-time of inactivation of 35 min. These data strongly suggest a very similar interaction in the two cases.

Thiol reducing agents such as dithiothreitol or β-mercaptoethanol affect the inactivation of coat protein by 5-bromouridine. When 10 mM DTT is included in an inactivation reaction similar to that in Figure 1, inactivation is not observed. In addition, although the inactive bromouridine–coat protein

Table III: Inactivation of Coat Protein by Nucleotides[a]

nucleotide	% act.	nucleotide	% act.
none	(100)	pppBrU	100
U	100	pFU	9
Up	100	pIU	3
pUp	100	C	90
BrU	1	5-azaC	9
BrUra	1	pBrC	1
pBrU	3	pIC	35
pBrUp	1	pBrA	67
BrdU	81	pBrG	100

[a] R17 coat protein (4 μM) was incubated with 80 mM nucleotide for 6 h at room temperature and then assayed for RNA binding activity.

Table IV: Reaction of Coat Protein with Sulfhydryl Reagents

	% act.	
[reagent]/[cysteine][a]	*N*-ethylmaleimide	*p*-(chloromercuri)benzenesulfonate
0	100	100
0.25	48	9
0.50	25	<1
1.00	22	<1
1.00 (+DTT)		100
2.00	13	<1
5.00	2	

[a] R17 coat protein has two cysteines (Weber, 1967).

complex is stable to 40-fold dilution and further incubation for 12 h, the presence of 10 mM DTT in the dilution buffer substantially reverses the inactivation (Figure 1). The reversal is comparatively rapid and is generally complete in 1 h, a result similar to that observed for tRNA synthetases (Starzyk et al., 1982).

A variety of nucleosides and nucleotides were tested for their ability to inactivate R17 coat protein (Table III). It is clear that an electron-withdrawing substituent at the 5-position of the pyrimidine ring promotes inactivation. Thus, while 5-bromouridine, 5-bromouracil, and the corresponding 5′-monophosphate and 3′,5′-bisphosphate all inactivate effectively, their unsubstituted counterparts do not. In addition, pBrC, pIC, and 5-azaC all inactivate coat protein more effectively than the corresponding unsubstituted molecules. The 8-bromopurines show little if any activity. It is interesting, however, that since BrdU does not inactivate coat protein very well, the presence of the 2′-hydroxyl appears important for inactivation. Finally, the inability of BrUTP to inactivate coat protein suggests that the degree of phosphorylation is also important for inactivation. Starzyk et al. (1982) also found substantial nucleotide specificity for the inactivation of tRNA synthetases although the exact specificity differs in comparison to that observed for R17 coat protein.

Thymidylate synthetase catalyzes the debromination of BrdUMP (Garrett et al., 1979), and tRNA synthetases catalyze deribosylation of BrU (Koontz & Schimmel, 1979). Coat protein was assayed under a variety of conditions for its ability to catalyze some conversion of BrU nucleosides. In the presence or absence of DTT, no protein-catalyzed alteration of BrU could be detected by HPLC analysis. Since this method of analysis is capable of detecting 5 or more turnovers per hour, R17 coat protein is therefore much less effective than the other enzymes in catalyzing the degradation of BrU.

R17 Coat Protein Contains an Essential Cysteine. Since it is generally believed that a cysteine is the protein nucleophile which forms a covalent bond with the pyrimidine (Wataya et al., 1980), we used *N*-ethylmaleimide and *p*-(chloromercuri)benzenesulfonate to probe for essential cysteines in

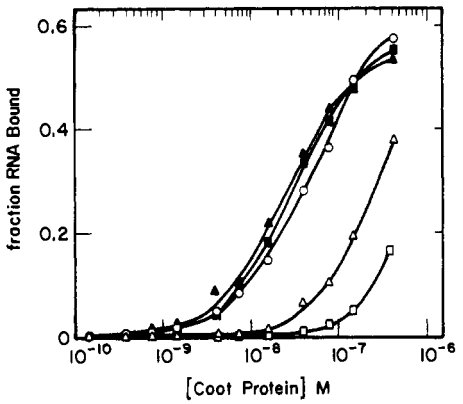


FIGURE 2: Assay of 21-mer binding to nucleotide-treated R17 coat proteins. R17 coat protein (10 μM) was incubated for 18 h in the absence (O) or presence of 80 mM pCp (triangles) or p-deazaUp (squares). The inactivated proteins were diluted 40-fold in the absence (open symbols) or presence (closed symbols) of 30 mM DTT and incubated an additional 2 h.

the coat protein. The results in Table IV show that a stoichiometric amount of *N*-ethylmaleimide inactivates R17 coat protein. This confirms the results of Berzin et al. (1981), who used disulfide reagents to show that Cys-46 of the closely related MS2 coat protein is essential for RNA binding. Both the relatively long reaction time and the slight molar excesses required to achieve complete inactivation suggest that the reactive cysteine might be somewhat buried in the protein structure. Consistent with this explanation is the observation that coat protein remains quite active in the absence of reducing reagents, and the RNA binding activity has absolutely no requirement for DTT or similar reagents (Carey et al., 1983a).

The reaction between R17 coat protein and *p*-(chloromercuri)benzenesulfonate also results in a loss of RNA binding activity. Complete inactivation is reached at substoichiometric amounts of the reagent (Table IV). As expected, full activity is restored if the inactivated protein is incubated with DTT. These results obtained with sulfhydryl modifying reagents suggest that at least one cysteine in the protein is in close proximity to the RNA binding site.

Inactivation of Coat Protein by Cytidine Nucleotides. The observation that substitution of a single uridine residue by cytidine resulted in an increase in affinity for protein binding (Carey et al., 1983), and the ability of 5-halopyrimidines to inactivate coat protein, led us to test cytidine nucleotides for coat protein inactivation. The effects of incubating R17 coat protein with pCp and p-deazaUp are shown in Figure 2. Both nucleoside bisphosphates are able to inactivate the protein, the greater effect being observed for the uridine analogue. In each case, dilution of the inactive complex followed by incubation with 30 mM DTT results in complete restoration of the RNA binding activity (Figure 2). Simply diluting the inactive complex and incubating in the absence of DTT do not reactivate the protein, as observed for BrU–coat protein complexes.

Inactivation of R17 coat protein by 3′-CMP is a pseudo-first-order kinetic process (Table I). As indicated, the 3′-CMP inactivation is approximately 10-fold slower in comparison to BrU inactivation measured under identical conditions. The faster rate of inactivation for BrU may well be the result of the electron-withdrawing substitution at the 5-position. At a fixed incubation time, the inactivation of coat protein by 3′-CMP is linearly dependent on the nucleotide concentration up to 160 mM (Table II). Thus, no saturation is observed.

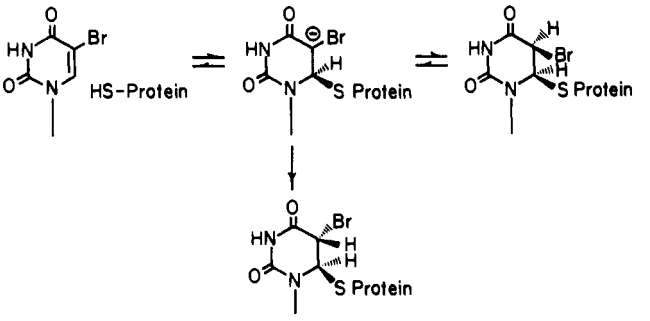
The results in Table V indicate that a variety of cytidine nucleotides are able to inactivate coat protein. The extent of

Table V: Cytidine Inactivation of Coat Protein[a]

nucleotide	% act.	nucleotide	% act.
none	100	pCp	7
U	100	p-deazaUp	2
C	67	C>p(2′,3′)	2
m^3C	100	BrU	33[b]
pC	52	pBrU	25[b]
Cp(3′)	47	pBrUp	5[b]
Cp(2′)	52		

[a] R17 coat protein (16 μM) was incubated with 80 mM nucleotide for 18 h at room temperature and then assayed for RNA binding activity. [b] Incubation conditions same as above, except that incubation time was 3.5 h at room temperature.

Scheme I: Possible Mechanisms for Inactivation of Coat Protein by BrU[a]



[a] The stereochemistry shown has been chosen arbitrarily.

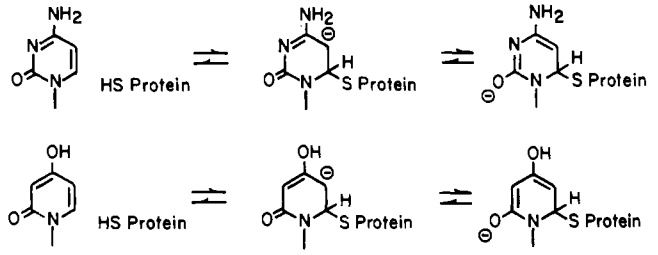
inactivation depends upon the phosphorylation state of the nucleoside. In general, the inactivation strength increases in the series N < Np ≃ pN < pNp < cyclic Np. The bromouridine nucleotides also appear to follow this series (Table V).

Discussion

In analogy to thymidylate synthetase (Poglotti & Santi, 1977) and tRNA synthetases (Starzyk et al., 1982), a model can be proposed for the inactivation of R17 coat protein by 5-halopyrimidines. As shown in Scheme I, a cysteine on the protein could attack the 6-carbon of the halopyrimidine ring, forming an extremely reactive carbanion at the 5-position. The carbanion would then rapidly abstract a proton from either the protein or the water, forming a dihydropyrimidine adduct with a chiral center at the 5-carbon. If the reaction results in the formation of a specific stereoisomer, reversal would occur by abstraction of the same proton from the same face of the ring. In the rare case where the dihydro adduct of opposite chirality is formed, bromine would occupy the site for proton abstraction, thereby preventing reversibility of the reaction (Scheme I). This irreversible complex would prevent subsequent binding of RNA to the protein. Starzyk et al. (1982) have proposed in the case of tRNA synthetases that the initial intermediate is converted to a dead-end product by displacement of bromine by a second nucleophilic attack. We are unable to distinguish between these possible mechanisms for the inactivation of R17 coat protein by 5-bromouridine. However, the ability of sulfhydryl modifying reagents to inactivate coat protein does suggest a central role for a cysteine side chain in the mechanism.

Although the mechanism suggests that any 5-halogenated pyrimidine would be able to inactivate R17 coat protein, substantial differences in the degree of inactivation were observed among the nucleotide analogues tested. While some of the differences could reflect the differential susceptibility of C6 to nucleophilic attack, it seems unlikely that all of these data can be explained this way. In particular, it is striking that 5-bromodeoxyuridine and 5-BrUTP are not able to in-

Scheme II: Possible Mechanism for Inactivation of Coat Protein by Cytidine and 3-Deazauridine



activate coat protein while 5-bromouridine and 5-bromouridine 3′,5′-bisphosphate are very effective. These data suggest that despite the absence of saturation with BrU, a specific binding event must occur between the protein and the nucleotide prior to the inactivation step. A similar conclusion was reached by Starzyk et al. (1982) for the tRNA synthetases.

The inactive complex formed between 5-bromouridine and coat protein can be reactivated rapidly by dithiothreitol and other reducing reagents. Reversal by dithiothreitol may occur by a mechanism known for the chemical debromination of 5-bromouridine by cysteine (Wataya et al., 1973) and believed to occur for tRNA synthetases (Starzyk et al., 1982). Alternatively, direct attack of the sulfhydryl on the protein–nucleotide bond could occur.

A number of cytidine nucleotides were also found to be inhibitors of R17 coat protein. Although the extent of inactivation is less, it appears to depend upon the phosphorylation state of the nucleotide in a manner similar to the bromouridine nucleotides. This suggests that both types of nucleotides are acting at the same site on the protein. While it might be argued in the case of the inactivation of coat protein by halopyrimidines that the electron-withdrawing substituent creates an enhanced site for nucleophilic attack by an essential cysteine on the external surface of the protein, this reasoning is less likely to account for the inactivation of coat protein by cytidine nucleotides. The 6-carbon is much less reactive in cytidine than in a halopyrimidine. Thus, CMP is unlikely to be a generalized sulfhydryl modifying reagent but rather must first bind to a specific site on the protein in order to be active.

Cytidine-inactivated coat protein is also quite stable to dilution followed by further incubation. Since cytidine is more susceptible than uridine to nucleophilic attack, it is possible that the equilibrium favors adduct formation. Alternatively, the stability could be explained by the resonance structure shown in Scheme II. The 2-keto group could act as a sink for the negative charge generated when nucleophilic attack occurs at the 6-position. Protonation could result or the structure could be stabilized if a positive charge on the protein forms an ionic bond to the enolate. The inactivation of coat protein by 3-deazauridine 3′,5′-bisphosphate is consistent with this latter mechanism. Replacement of the 3-nitrogen in the uridine ring with a carbon gives rise to a hydroxyl at C4 and a conjugated system analogous to that found in cytidine (Schwalbe et al., 1972; Schwalbe & Saenger, 1973). Inactivation could then occur by the same mechanism as drawn for cytidine.

Cytidine-inactivated coat protein is readily reactivated by dithiothreitol. Since cytidine does not have a good leaving group at the 5-position, reversal is unlikely to occur by attack of a sulfhydryl at C5. Further investigation will be required to understand the mechanism by which DTT can reverse CMP-inactivated coat protein.

The nucleotide inactivation data reported here strongly suggest that a similar Michael addition reaction occurs between R17 coat protein and one of the two single-stranded uridines on the RNA. Since substitution of one of the uridines with other nucleotides has little effect upon the K_d of the RNA–protein interaction, it is likely that the other uridine is the one involved (Carey et al., 1983). Protein-catalyzed ^3H exchange data on the 21-mer would provide direct conformation of this site. It is, however, striking that substitution of the uridine by a cytidine at this position in the 21-mer leads to a substantial increase in K_a. The relation of this observation to the CMP inactivation of coat protein is not clear, however. The CMP–coat protein complex appears to be much more stable than the complex of C-containing 21-mer bound to coat protein. The example, CMP-inactivated coat protein can be diluted 40-fold and incubated for up to 24 h at room temperature and show no detectable reactivation. In contrast, the dissociation rate of the C-containing 21-mer complex to coat protein after a similar dilution at 24 °C is about 10 min (P. Lowary, unpublished results). It is possible that the adjacent nucleotides in the 21-mer alter the equilibrium of the Michael addition reaction.

In the case of enzyme–nucleotide interactions, the formation of a covalent protein–nucleotide bond serves to activate a site on the substrate for further reactivity. This is unlikely to be the purpose of covalent bond formation in RNA–protein interactions. Here, the covalent bond formed must readily reverse to allow for a dynamic equilibrium. It would be interesting to know the relative contribution of such a transient covalent bond to the overall free energy of formation of the protein–RNA complex. Substitution of the sensitive uridine in the 21-mer by modified nucleosides might allow us to measure this effect for the R17 complex.

Registry No. BrU, 957-75-5; BrUra, 51-20-7; pBrU, 2149-79-3; pBrUp, 68351-45-1; BrdU, 59-14-3; pFU, 796-66-7; pIU, 7531-46-6; C, 65-46-3; 5azaC, 320-67-2; pBrC, 4181-56-0; pIC, 7531-47-7; pBrA, 23567-96-6; 3′CMP, 84-52-6; L-cysteine, 52-90-4; cytosine, 71-30-7; pyrimidine, 289-95-2.

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Transport of α- and β-D-Glucose by the Intact Human Red Cell†

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ABSTRACT: The kinetics of α- and β-D-glucose mutarotation and the transport of these anomers by intact human red cells were determined at 0.6 and 36.6 °C. The mutarotation coefficients for α- and β-D-glucose in cell-free tris(hydroxymethyl)aminomethane medium (pH 7.4) at 0.6 °C are (2.25 ± 0.2) and (1.73 ± 0.42) × 10^{-3} min^{-1}, respectively, and at 36.6 °C are (69 ± 12) and (75 ± 5) × 10^{-3} min^{-1}, respectively. These values are in good agreement with previous estimates. At 0.6 °C, the red cell contains no detectable mutarotase activity. Initial rates of sugar uptake were measured by using radiolabeled D-glucose and time courses of uptake by turbidimetry. The time courses of α- and β-D-glucose and an equilibrium mixture of α- and β-D-glucose infinite-cis entry are identical at 0.66 °C (n = 41) where negligible mutarotation is observed. The apparent K_i values for inhibition of radiolabeled D-glucose initial uptake by unlabeled α- or β-D-glucose at 0.6 °C are identical (1.6 mM). The calculated V_{max} parameters for uptake of the radiolabeled anomers at this temperature are also indistinguishable. The time courses of infinite-cis α- and β-D-glucose uptake at 36.66 °C are identical (n = 40). While D-glucose mutarotation is more rapid at this temperature, the anomers of D-glucose are not transported differently by the red cell hexose transfer system. These findings confirm the suitability of the use of the integrated rate analysis for infinite-cis entry kinetics and support rejection of the symmetric and asymmetric carrier models for red cell sugar transport [see Naftalin, R. J., & Holman, G. D. (1977) in *Membrane Transport in Red Cells* (Ellory, J. C., & Lew, V. C., Eds.) pp 257-300, Academic Press, New York].

Hexose transfer in intact human erythrocytes displays kinetic asymmetry (Widdas, 1980). K_m and V_m for D-glucose net exit are some 10-fold greater than K_m and V_m for influx (Geck, 1971; Regen & Tarpley, 1974; Baker & Widdas, 1973). Moreover, when the external hexose transfer sites are saturated with substrate, a second, kinetically distinct (high affinity, low K_m) transport site is detected at the inner surface of the plasma membrane both in infinite-cis entry experiments (Hankin et al., 1972; Ginsburg & Stein, 1975; Foster et al., 1979; Carruthers & Melchior, 1983a) and in infinite-trans exit determinations (Baker & Naftalin, 1979). These measurements were made either by analysis of initial rates of radiolabeled sugar entry and exit (Ginsburg & Stein, 1975; Baker & Naftalin, 1979) or by transformation of the time course of net sugar uptake according to the integrated form of the infinite-cis entry Michaelis–Menten equation (Hankin et al., 1972; Ginsburg & Stein, 1975; Foster et al., 1979; Carruthers & Melchior, 1983a) and thus indicate that the obtained results are independent of both the method of measurement and the method of kinetic analysis. These observations are incompatible with the asymmetric form of the simple mobile carrier model for red cell sugar transport (Widdas, 1952; Regen & Tarpley, 1974).

Gorga & Lienhard (1981) suggested a possible explanation for these findings—that α and β anomers of D-glucose are transported with different velocity constants (V_m) or with different affinities (K_m) by the red cell hexose transfer system. Under experimental conditions, the red cell may be exposed to a mixture of these anomers (the equilibrium ratio is 36.2:63.8 α:β; Pigman & Anet, 1972). Hence, analysis of hexose transfer must recognize both parallel uptake and competition for uptake between these anomers. This would mean that the steady-state analyses of hexose transfer (both initial *and* integrated Michaelis–Menten rate equations) employed in the above studies were not, in the most rigorous sense, valid. If correct, this suggestion has serious analytical implications. Nevertheless, this suggestion still fails to account for the two quite different transport sites detected at the interior of the cell under zero-trans and infinite-trans exit conditions. Moreover, it is not clear how differential transport of anomers can account for the loss of hexose transfer kinetic asymmetry upon removal of cellular contents (Carruthers & Melchior, 1983a).

The simplest test of the anomer hypothesis is to determine the kinetics of α- and β-D-glucose uptake by the red cell under

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