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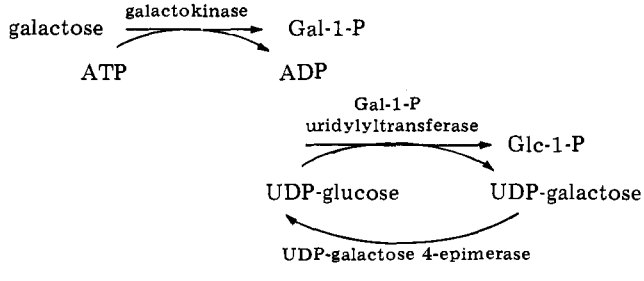
Uridine Diphosphate Galactose 4-Epimerase: Nucleotide and 8-Anilino-1-naphthalenesulfonate Binding Properties of the Substrate Binding Site†

Shan S. Wong and Perry A. Frey*

ABSTRACT: *Escherichia coli* UDP-galactose 4-epimerase in its native form (epimerase·NAD) binds 8-anilino-1-naphthalenesulfonate (ANS) at one tight binding site per dimer with a dissociation constant of 25.9 ± 2.1 μM at pH 8.5 and 27 °C. This appears to be the substrate binding site, as indicated by the fact that ANS is a kinetically competitive reversible inhibitor with a K_i of 27.5 μM and by the fact that ANS competes with UMP for binding to the enzyme. Upon binding at this site the fluorescence quantum yield of ANS is enhanced 185-fold, and its emission spectrum is blue shifted from a λ_{max} of 515 to 470 nm, which suggests that the binding site is shielded from water and probably hydrophobic. Competitive binding experiments with nucleosides and nucleotides indicate that nucleotide binding at this site involves coupled hydrophobic and electrostatic interactions. The reduced form of the enzyme (epimerase·NADH) has no detectable binding affinity for ANS. The marked difference in the affinities of the native and reduced enzymes for ANS is interpreted to be a manifestation of a conformational difference between these enzyme forms.

UDP[1]-galactose 4-epimerase, which catalyzes the interconversion of UDP-galactose and UDP-glucose, is one of the three enzymes of the Leloir pathway for the conversion of galactose to Glc-1-P. This pathway is the major route by which nutrient galactose enters the energy-producing metabolism of most organisms. The other two enzymes of the pathway are galactokinase and galactose-1-phosphate uridylyltransferase, which by coupled action convert galactose to UDP-galactose in preparation for the action of the epimerase. The Leloir pathway is illustrated by Scheme I. Another important function of UDP-galactose 4-epimerase is the net production of UDP-galactose from UDP-glucose under conditions in which galactose is not available as a nutrient but galactosyl units are needed for the biosynthesis of cellular constituents such as glycoproteins. UDP-glucose is produced under these conditions

SCHEME I.



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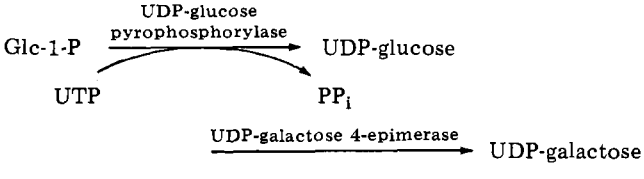

primarily by the action of UDP-glucose pyrophosphorylase on Glc-1-P and UTP. This is illustrated by Scheme II.

The *E. coli* UDP-galactose 4-epimerase is a dimer of identical subunits which contains one molecule of very tightly

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; NAD, nicotinamide adenine dinucleotide; UMP, UDP, and UTP, uridine 5′-mono-, di-, and triphosphates; AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphates.

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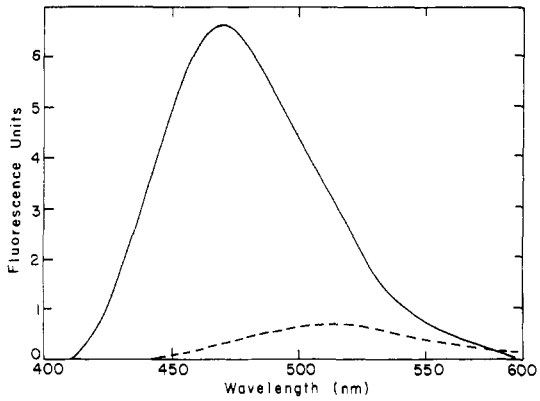

FIGURE 1: Fluorescence emission spectra of ANS and epimerase·NAD·ANS complex. The more intense spectrum is that of a solution of 13.8 μM UDP-galactose 4-epimerase containing 0.45 μM ANS at pH 8.5. The excitation wavelength was 375 nm, and the spectrum shown has been corrected for a low level of fluorescence due to the presence of a small amount of epimerase·NADH in the epimerase·NAD. The less intense spectrum is that of a 4.5 μM concentration of free ANS in the absence of enzyme.

bound NAD per dimer, and this NAD functions as a true coenzyme in the catalytic process (Wilson and Hogness, 1964, 1969). The *S. fragilis* enzyme also contains one NAD per two subunits (Darrow and Rodstrom, 1968).

Very little information is available on the nature of the binding interactions between the nucleotide portions of substrates and the active sites of UDP-galactose 4-epimerases, although this binding is considered to be a decisively important part of the mechanism of action of the *E. coli* and *S. fragilis* enzymes (Kang et al., 1975; Wong and Frey, 1977).

In the first place, the binding interactions between these enzymes and uridine nucleotides appear to be coupled to important enzyme conformational transitions, as evidenced by the fact that uridine nucleotides are essential activators of the reductive inactivations of these enzymes to epimerase·NADH by sugars such as glucose and by $NaBH_3CN$ (Bhaduri et al., 1965; Bertland, et al., 1966; Bertland and Kalckar, 1968; Kalckar et al., 1970; Davis et al., 1974). The *E. coli* enzyme has been most thoroughly studied in this respect, and the kinetics for UMP-dependent reduction by glucose and of UDP-glucose activated reduction by D-fucose are most consistent with the interpretation that the binding of the nucleotides activates the reduction of tightly bound NAD by inducing a conformational change in the protein structure (Kang et al., 1975; Blackburn and Ferdinand, 1976). This change is probably at least in part a change in secondary structure (Wong et al., 1977).

Secondly, in the case of the *E. coli* enzyme it is known that the overwhelmingly major part of the substrate binding free energy involves the nucleotide components of substrates (Wong and Frey, 1977). This and the fact that the binding of sugar moieties of substrates is weak constitute the experimental basis for the proposed mechanism of nonstereospecific hydrogen transfer, i.e., epimerization, catalyzed by this enzyme (Kang et al., 1975; Wong and Frey, 1977). It is postulated that the uridine nucleotide serves an essential catalytic role as the strong binding component of substrates. Thus, upon binding, the uridylpyrophosphoryl moiety firmly anchors the glycosyl component to the active site without the necessity for strong, highly specific binding of the sugar. In this way the weak and relatively nonspecific binding interactions at the glycosyl locus, which are required for nonstereospecific hydrogen transfer by NAD in the active site, are realized while maintaining strong overall binding via the anchoring effect of the nucleotide component.

In this paper we report the results of studies probing the nature of binding interactions between the *E. coli* epimerase active site and uridine nucleotides. We also compare the binding of uridine nucleotides and 8-anilino-1-naphthalenesulfonate (ANS) at this site.

Materials and Methods

Enzymes. E. coli UDP-galactose 4-epimerase was purified from the regulatory mutant ATCC 27797 according to the procedure of Wilson and Hogness (1964) as modified in the hydroxylapatite chromatography step (Wong and Frey, 1977). UDP-glucose dehydrogenase from beef liver was purchased from Sigma Chemical Co. The epimerase·NADH·UMP complex was prepared as described by Wong et al. (1978).

Chemicals. Uracil, uridine, UMP, UDP, UTP, adenosine, AMP, ADP, and ATP as well as ANS (NH_4^+ salt) and bicine were purchased from Sigma Chemical Co. ANS was repurified by recrystallization from hot water following clarification with charcoal. The nucleotides, nucleosides, and nucleotide bases were used without further purification.

Assays. In the initial rate kinetics the rates were measured by a two-step assay method. UDP-galactose at concentrations between 0.05 and 0.7 mM was incubated with UDP-galactose 4-epimerase at a suitable assay concentration in 0.1 M sodium bicinate buffer at pH 8.5 and 27 °C for 5 min. The reactions were stopped by heating at 100 °C for 3 min and the amount of UDP glucose formed was measured as one half of the NADH produced after addition of NAD and UDP-glucose dehydrogenase. The factor of one half arises from the fact that the oxidation of UDP-glucose to UDP-glucuronate consumes 2 mol of NAD and produces 2 mol of NADH per mol of nucleotide sugar oxidized.

The concentration of UDP-galactose 4-epimerase used in binding experiments was measured spectrophotometrically at 280 nm assuming $A_{280} = 1.05$ for a 1.0 mg/mL solution as reported by Wilson and Hogness (1964). The concentrations of stock ANS solutions used in binding and kinetics experiments were measured spectrophotometrically at 350 nm using the extinction coefficient 6.3×10^3 M^{-1} cm^{-1} reported by Kolb and Weber (1975).

Binding Experiments. All binding experiments were carried out in 0.1 M sodium bicinate buffer at pH 8.5 and 27 °C. Binding of ANS by UDP-galactose 4-epimerase was studied by the flow-dialysis technique introduced by Colowick and Womack (1969) using the cell and technique described by Klapper (1970). Binding of ANS, nucleotides, nucleosides, nucleotide bases, and anions was studied by fluorescence techniques described in the text using a Perkin-Elmer MFP spectrofluorometer.

Results

Inhibition of UDP-Galactose 4-Epimerase by ANS. ANS was evaluated as an inhibitor of *E. coli* UDP-galactose 4-epimerase. It was found to be a very effective inhibitor, more effective than uridine nucleotides such as UMP whose inhibition constants are between 0.2 and 1.5 mM (Wong and Frey, 1977). An analysis of the inhibition kinetics at ANS concentrations of 10.1, 30.2, and 65.8 μM showed that the inhibition is cleanly competitive with respect to UDP-galactose, and the competitive inhibition constant was found to be 27.5 μM. We concluded from this that the bindings of ANS and UDP-galactose by this enzyme are mutually exclusive.

Fluorescence Properties of Enzyme-Bound ANS. The solid-line spectrum in Figure 1 is the fluorescence emission spectrum of ANS bound to epimerase and the dashed line

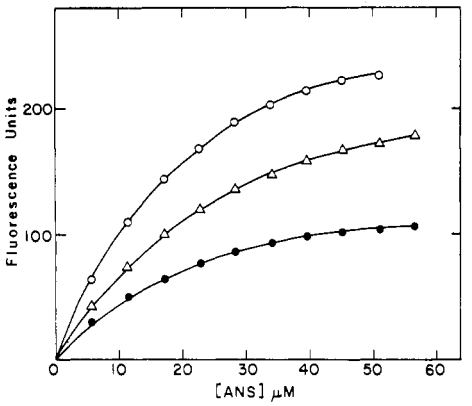

FIGURE 2: Fluorescence data on binding of ANS by epimerase·NAD. Each curve was generated by titrating a solution of epimerase at pH 8.5 and 25 °C with additions of ANS at the indicated concentrations and measuring the florescence emission at 470 nm upon excitation at 375 nm. The data were corrected for the fluorescence due to free ANS: (●–●) 1.4 μM epimerase·NAD; (○–○) 2.8 μM epimerase·NAD; (△–△) 2.8 μM epimerase·NAD plus 0.4 mM UMP.

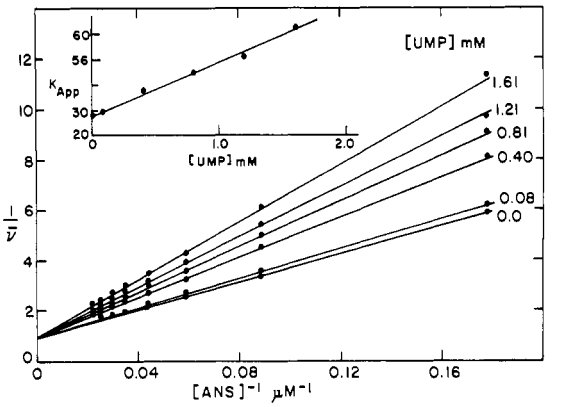

FIGURE 3: Number of ANS binding sites and competitivity with UMP. Titration data were obtained under the conditions of Figure 2 in the absence of UMP and at five different UMP concentrations. The fluorescence data were used to calculate $\bar{\nu}$ values which were used in the double-reciprocal plot. The UMP concentrations used are given in the figure. The inset is the slope replot used to evaluate the dissociation constants of ANS and UMP.

spectrum is that of free ANS at ten times larger concentration under otherwise identical conditions. The fluorescence quantum yield for ANS in epimerase·ANS can be calculated from the data in Figure 1 by a procedure described by Parker and Rees (1960) if the quantum yield for free ANS is known. Using the value 0.004 reported by Weber and Young (1964) for free ANS, we calculate a quantum yield of 0.75 for epimerase·ANS. As shown in Figure 1, the emission maximum for epimerase·ANS is blue shifted from 515 to 470 nm relative to that for ANS. These comparisons indicate that at least one ANS binding site is hydrophobic (Weber and Laurence, 1954; Stryer, 1968), approximately as hydrophobic as the tight ANS binding sites of bovine serum albumin (Kolb and Weber, 1975), but less than that of apomyoglobin (Stryer, 1968). In addition to giving this information about the binding site, these properties also facilitate the measurement of dissociation constants for ANS and other competitive inhibitors, because the fluorescence emission of free ANS at 470 nm is insignificant relative to that of epimerase·ANS.

Stoichiometry of ANS Binding. The number of ANS binding sites and the dissociation constant for ANS were measured by two independent techniques, both of which showed that there is one tight binding site for ANS on each enzyme dimer and that the dissociation constant for ANS at this site is 27–28 μM.

Figure 2 depicts sample binding data obtained by measuring the fluorescence emission of solutions of epimerase and ANS as a function of ANS concentration at two different enzyme concentrations and in the presence and absence of UMP, which is a competitive reversible inhibitor (Wong and Frey, 1977). The data show that the observed fluorescence is directly proportional to enzyme concentration at any ANS concentration and that the fluorescence is quenched by the presence of UMP.

Inasmuch as both ANS and UMP are kinetically competitive reversible inhibitors of the enzyme, it is reasonable to expect them to bind competitively. If the ANS-binding sites detected fluorimetrically in Figure 1 are also those which bind UMP, the quenching of fluorescence by UMP in Figure 2 can be understood on the basis that UMP displaces ANS from the enzyme by competition. That this is quantitatively the case is shown by Figure 3, where the reciprocal of the fraction of occupied sites is plotted vs. the reciprocal of ANS concentration, both in the absence of UMP and in the presence of five different UMP concentrations. The lines converge on the ordinate

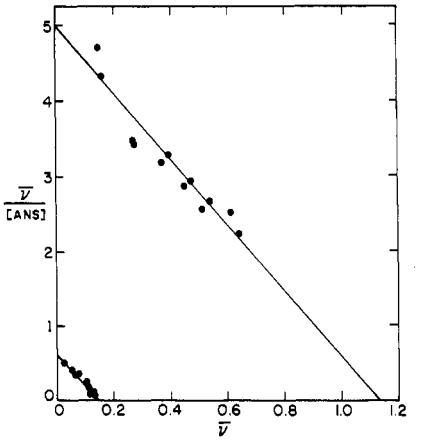

FIGURE 4: Analysis of ANS binding by the flow dialysis technique. The flow-dialysis procedure is described under Materials and Methods. The enzyme concentration was 138 μM, and $\bar{\nu}$ values were calculated from the A_{350} measurements of [ANS] in the flow system. The measurements were made in 10 mM potassium phosphate at pH 8.5 in the presence of 1 mM EDTA. The plot in the lower left of the figure was obtained on epimerase·NADH·UMP under identical conditions. The fractional binding observed is attributed to the presence of a small amount of epimerase·NAD.

scale, showing that ANS and UMP binding are competitive. The significance of the convergence at 1.0 on the ordinate scale is that there is only one fluorimetrically detectable binding site per enzyme dimer. The line corresponding to the absence of UMP in Figure 4 is plotted according to the reciprocal of the Klotz equation (Klotz, 1946):

$$\frac{1}{\bar{\nu}} = \frac{1}{n} + \frac{K_D}{n[ANS]} \quad (1)$$

in which $\bar{\nu}$ is the fraction of occupied sites, n is the number of equivalent, noninteracting binding sites per enzyme dimer (mol wt 79 000), and K_D is the dissociation constant for ANS. Since from Figure 3 the value of n is 1.0 and UMP competes with ANS for binding to the enzyme, the simplest binding model in quantitative accord with Figure 3 is given by eq 2 and 3 below, where K_D and K_U are the dissociation constants for ANS and UMP, respectively.

$$E + ANS \overset{K_D}{\rightleftharpoons} E\cdot ANS \quad (2)$$

$$E + UMP \overset{K_U}{\rightleftharpoons} E\cdot UMP \quad (3)$$

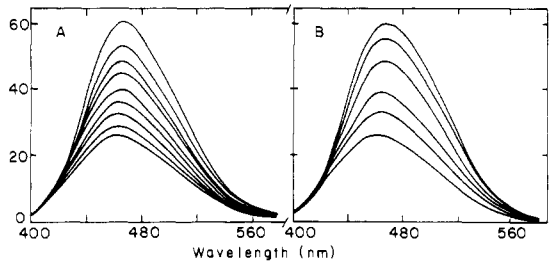

FIGURE 5: Reversible quenching of epimerase·NAD·ANS fluorescence by UMP. Part A. The solution contained initially 3.4 μM epimerase·NAD and 11.3 μM ANS in 0.1 M sodium bicinate buffer at pH 8.5 and 25 °C. The most intense emission spectrum in part A was obtained on this solution upon excitation at 375 nm. The succeeding quenched spectra were obtained upon addition of UMP at the following concentrations: 0.081, 0.16, 0.24, 0.40, 0.56, 0.81, 1.21, and 1.61 mM. Part B. The solution was back-titrated by the addition of ANS to the following concentrations: 11.3, 17.0, 22.6, 33.9, 45.2, and 56.5 μM.

All of the data are correlated by eq 4 derived from the foregoing binding model.

$$\frac{1}{\bar{\nu}} = 1 + \frac{K_D}{[ANS]}\left(1 + \frac{[UMP]}{K_U}\right) \quad (4)$$

The values for the binding parameters obtained from the slope replot in Figure 3 are K_D = 27.6 μM and K_U = 1.3 mM. These values are the same as the competitive inhibition constants, indicating that the fluorimetrically detected sites are the same as or overlap the nucleotide binding subsite of the active site.

Figure 4 is a Scatchard plot depicting ANS binding data obtained by the flow-dialysis technique. The upper plot refers to ANS binding to the native enzyme. The slope and intercept of this line give values of 1.13 binding sites per enzyme dimer with a dissociation constant of 22.6 μM, in good agreement with Figure 3. Since binding in this experiment was measured spectrophotometrically at the absorption maximum for ANS of 350 nm, all ANS binding sites would have been detected irrespective of whether they caused enhancement of ANS fluorescence. The results show that the only tight binding site is that detected in Figures 1–3, the site that produces enhancement of ANS fluorescence.

The plot in the lower right-hand corner of Figure 4 depicts one of several attempts to detect ANS binding to the epimerase·NADH·UMP complex. The data show binding to only 0.15 site per enzyme dimer, and the dissociation constant for this process is 28.5 μM, the same as that for epimerase·NAD. Inasmuch as the epimerase·NADH·UMP complex normally contains some epimerase·NAD because of autoxidation during isolation, this small amount of binding can be attributed to native enzyme. Despite repeated attempts, we have been unable to detect binding of ANS by epimerase·NADH. Although ANS and UMP compete for binding to epimerase·NAD, epimerase·NADH does not seem to bind ANS.

Displacement of ANS by Competitive Inhibitors. The 185-fold enhanced fluorescence of bound ANS and the fact that it binds to only a single site in competition with substrates and other competitive inhibitors makes it a convenient probe for studying the binding of competitive inhibitors of this enzyme. Such inhibitors quench the fluorescence of solutions of epimerase and ANS by displacing ANS from its binding site on the enzyme. This is illustrated in Figure 5, in which additions of UMP are shown to quench the fluorescence progressively. Quenching is fully reversed by further additions of ANS. Such data can be plotted according to eq 5 (Wong and Frey, 1977)

TABLE I: Relative Dissociation Constants of Nucleosides and Nucleotides.[a]

compd	K_{dissoc} (mM)
uracil	undetect
uridine	400[b]
UMP	1.9
UDP	1.6
UTP	4.3
adenosine	86
AMP	34
ADP	77
ATP	700[b]
sulfate	1600[b]
phosphate	1500[b]

[a] Solutions containing 35 μM UDP-galactose 4-epimerase and 11 μM ANS in 0.1 M sodium bicinate buffer at pH 8.5 were titrated with the indicated compounds as described in Figure 5. The dissociation constants were evaluated by plotting the data according to eq 5 as described in the text. [b] Estimated values based on slight quenching of epimerase·NAD·ANS fluorescence.

$$\frac{[UMP]}{1-\theta} = \frac{[ANS]}{K\theta} + \left(1 - \frac{1}{K}\right)[E{\cdot}ANS] \quad (5)$$

in which [UMP] and [ANS] are the total concentrations, θ is the ratio of fluorescence in the presence and absence of UMP, $K = K_D/K_U$, and [E·ANS] is the initial total concentration in the absence of UMP.

A plot of $[UMP]/(1-\theta)$ vs. $1/\theta$ yields a straight line with a slope of $[ANS]K_U/K_D$. Since [ANS] and K_D for ANS are known, K_U can be calculated from the slope.

When this was done with the data in Figure 5, the value obtained for K_U was 1.86 mM, in agreement with the values obtained kinetically (Kang et al., 1975; Wong and Frey, 1977) and by other techniques utilized in this work.

Dissociation constants measured by the foregoing technique for a number of compounds are given in Table I. They show that nucleotides are much more effectively bound than nucleosides or sulfate or phosphate dianions. It is interesting that uridine nucleotides are much more tightly bound than adenine nucleotides, while adenosine is significantly more tightly bound than uridine. Bindings of sulfate, phosphate, and the free bases adenine and uracil are essentially undetectable at accessible or reasonable concentrations. The data show that binding interactions to nucleotides involve the heterocyclic bases, the ribosyl groups, and the phosphoryl or pyrophosphoryl groups. These interactions must be coupled in some way because we are unable to detect the binding of phosphate dianion, although UMP dianion is bound 200 times more tightly than uridine.

Although ANS could be displaced from its binding site by nucleotides, it could not be displaced by 1 M glucose. Therefore, while the ANS binding site may involve the nucleotide binding subsite of the active site, it evidently does not overlap the glycosyl binding subsite.

Contrasting Properties of ANS and UMP as Epimerase Ligands. The preceding results show that ANS and UMP compete for binding to epimerase·NAD. Competitivity is consistent with the view that ANS and UMP bind at the same site on epimerase·NAD. This relationship between ANS and UMP does not, however, extend to other aspects of the binding interactions between epimerase and uridine nucleotides. It is well known that UMP activates the reduction of epimerase·NAD to the epimerase·NADH·UMP complex by sugars and borohydrides; however, ANS does not promote the reduction of epimerase·NAD by these reducing agents. It is also known

that epimerase·NADH is very labile to autoxidation but is stabilized by uridine nucleotides such as UDP-sugars and UMP; however, ANS does not stabilize epimerase·NADH against autoxidation. As mentioned in a preceding section, we cannot detect any binding of ANS by epimerase·NADH using the flow-dialysis technique, showing that epimerase·NADH has at best a very low-binding affinity for ANS.

The fact that ANS does not stabilize epimerase·NADH suggested another experimental approach by which epimerase·NADH might be observed to interact with ANS, even if the binding were weak. It has been shown that uridine nucleotides readily exchange with UMP in the epimerase·NADH·UMP complex and that this exchange proceeds according to eq 6 and 7 (Wee and Frey, 1973; Wong and Frey, 1977).

$$\text{E·NADH·UMP} \rightleftharpoons \text{E·NADH} + \text{UMP} \quad (6)$$

$$\text{E·NADH} + \text{nucleotide} \rightleftharpoons \text{E·NADH·nucleotide} \quad (7)$$

If ANS were to exchange with UMP in this way, the product should be epimerase·NADH·ANS, which could be expected to undergo oxidation by O_2 to epimerase·NAD, since ANS does not stabilize the reduced complex against autoxidation. Therefore, if ANS can exchange with UMP in epimerase·NADH·UMP, this should be observed as a large decrease in NADH fluorescence upon combining this complex with a large [ANS].

We have made several attempts to detect such an effect, but we have never observed a decrease in the fluorescence of epimerase·NADH·UMP upon adding up to 2.4 mM ANS in the presence of 40 μM UMP. We have been unable to observe the slightest indication of any binding interaction between epimerase·NADH and ANS.

Discussion

The data in Table I show that the binding of nucleotides at the active site of *E. coli* UDP-galactose 4-epimerase involves interactions of the heterocyclic base, the ribosyl, and the phosphoryl or pyrophosphoryl moieties of nucleotides with the nucleotide-binding region of the active site. The binding process apparently involves hydrophobic and electrostatic interactions and probably hydrogen bonding as well. No one of these completely dominates the binding process, as shown by the fact that no binding of phosphate or heterocyclic bases themselves can be detected although both are clearly important in nucleotide binding. Moreover, the structure of ANS incorporates both hydrophobic and electrostatic character, and this molecule is very tightly bound by the enzyme, which further supports the idea that electrostatic and hydrophobic forces are involved. They are coupled, however, probably by a chelate effect, so that neither is dominant and both are required for strong binding.

Competitivity and the probable involvement of hydrophobic and electrostatic interactions are the only properties in common between uridine nucleotides and ANS as ligands for the enzyme. While it is competitive with substrates and UMP, ANS differs radically from substrates and UMP in that (a) it is much more tightly bound by epimerase·NAD than are uridine nucleotides; (b) it does not activate reduction of epimerase·NAD to epimerase·NADH as do uridine nucleotides; (c) it does not protect epimerase·NADH against autoxidation as do uridine nucleotides; and (d) epimerase·NADH does not appear to have any binding affinity for ANS, although this form of the enzyme binds uridine nucleotides 100- to 1000-fold more tightly than does epimerase·NAD. These aspects of enzyme–ANS binding are consistent with the existence of at least two conformational states for this enzyme. These states have been postulated on the basis of the effects of uridine nucleotides on the redox properties of epimerase·NAD, on the basis of the fact that epimerase·NADH binds uridine nucleotides much more tightly than does epimerase·NAD, and on the basis of a difference in the CD spectra of epimerase·NAD and epimerase·NAD·UMP (Wong et al., 1977). The contrasting properties of ANS listed above complement these findings on uridine nucleotide binding and further support the postulate.

TABLE II: Proposed Binding Interactions and Enzyme Conformational Transitions.

	K_{dissoc}	ref
E·NAD·ANS ⇌ E·NAD + ANS	0.025 mM	this work
E*·NAD·UMP ⇌ E·NAD + UMP	1.5 mM	Wong and Frey, 1977
E*·NADH·UMP ⇌ E*·NADH + UMP	0.012 mM	Wong and Frey, 1977
(E*·NADH·ANS) ⇌ E*·NADH + ANS	>10 mM	this work

The proposed binding interactions and enzyme conformational transitions are depicted in the binding model of Table II, in which E·NAD symbolizes the native enzyme, E*·NAD is a conformationally altered form of the enzyme, and UMP is a representative nucleotide. E·NAD and E*·NADH·UMP are known to differ in protein secondary structure and UMP binding activates the reduction of E·NAD to E·NADH by sugars and borohydrides, presumably by forcing a protein conformational change to E*·NAD·UMP upon binding. The E* conformation is also stabilized by the reduction of bound NAD to NADH as shown by the fact that E*·NADH binds UMP with more than 100 times the affinity of E·NAD. Note that ANS binding is not coupled to the E to E* transition, and E*·NADH·ANS is represented parenthetically because it has not been observed.

The present work further supports the proposed existence of two conformational states by showing that the native enzyme binds ANS much more tightly than UMP while the reduced form has no detectable binding affinity for this compound. Apparently, the nucleotide binding site in epimerase·NAD is physicochemically more complementary to ANS than to uridine nucleotides, while this site in the conformationally altered E* form in E*·NADH is physicochemically more complementary to uridine nuclotides than to ANS.

We cannot rigorously exclude the possibility that the ANS binding site is physically separated from the active site. If this turns out to be the case, the sites must interact in such a way that ligand-induced structural changes at one site lead, via protein conformational mobility, to structural changes at the other site which are of such a nature as to block binding of the other ligand at that site. This would in no way alter the basic significance of our ANS binding data, that is, that the enzyme exists in at least two conformational states.

Acknowledgment

We are grateful to Professor M. H. Klapper for making his spectrofluorometer available for our use.

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Purification and Characterization of Canine α-1-Antiproteinase†

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ABSTRACT: The principal canine plasma protease inhibitor, α-1-antiproteinase, has been purified 90-fold with a 25% yield to apparent homogeneity. The purification scheme includes anion-exchange chromatography, to separate away the bulk of the serum albumin; affinity chromatography by insolubilized concanavalin A, to remove most of the other serum proteins as well as traces of albumin; and, finally, sizing on Sephacryl-S-200. Unique to this purification scheme is the batch use of insolubilized hemoglobin–Sepharose beads to remove the ubiquitous contaminant haptoglobin. The purified material has an apparent molecular weight of 58 000, 11.2% carbohydrate, and an $E_{280nm}^{1\%} = 5.82$, and can be separated by isoelectric focusing into at least two distinct forms with p*I* values of 4.40 and 4.52. In addition, canine α-1-antiproteinase is immunologically distinct from human α-1-antiproteinase.

Over the past several years this laboratory has been working with a homologous animal model system for the study of the etiology of experimental canine emphysema (Marco et al., 1971; Mass et al., 1972; Weinbaum et al., 1974; Janoff et al., 1977). During the course of these studies it became necessary to purify canine α-1-antiproteinase (AP)[1] to serve as an antigenic and biochemical standard for differentiation of the various protease inhibitors found in the dog lung (Weinbaum et al., 1976).

Biochemically, AP is unusual because it is approximately twice the size of the serine proteases (i.e., trypsin and elastase) that it inhibits. It is difficult to purify in large quantities free from serum albumin, transferrin, and haptoglobin, although it is found in the normal dog at an abundant level of 150–200 mg/100 mL of serum (Kueppers, 1977, personal communication). Its presence is ubiquitous throughout the body as a normal component of serum as well as being associated with cell types such as platelets (Nachman and Harpel, 1976; Nalli et al., 1977) and macrophages (Cohen, 1973). In general terms, it is believed to be a broad spectrum inhibitor which modulates protease activities in conjunction with α-2-macroglobulin (Ohlsson, 1971).

In recent years, several different techniques have been applied to the purification of human AP, i.e., ion-exchange chromatography (Crawford, 1973; Chan et al., 1973; Berninger and Mathis, 1976; Hercz and Barton, 1977; Myerowitz et al., 1972a), affinity and gel-filtration chromatography (Liener et al., 1973; Murthy and Hercz, 1973; Travis et al., 1976), or thiol interchange chromatography (Laurell et al., 1975, 1977). The work reported in this paper draws in part from all these techniques. This paper presents a simple, rapid, large-scale purification of canine AP to homogeneity with high yield. In addition, data are presented which suggest that canine AP consists of at least two isoinhibitors.

Experimental Procedure

Materials

Chemicals and enzymes were obtained as follows: hemoglobin, benzamidine, polybrene (1,5-dimethyl-1,5-diazauradecamethylene polymethobromide), 2-mercaptoethanol, 1-*O*-methyl α-D-glucopyranoside, transferrin (human), aldolase, and ovalbumin were from Sigma Chemical Co.; con-

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Boc-Ala-ONp, *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester; BAPA, *N*-benzoyl-DL-arginine *p*-nitroanilide; AP, α-1-antiproteinase; EDTA, (ethylenedinitrilo)tetraacetic acid.

0006-2960/78/0417-3556$01.00/0