Effects of ATP and CTP on the Conformation of the Regulatory Subunit of *Escherichia coli* Aspartate Transcarbamylase in Solution: A Medium-Resolution Hydrogen Exchange Study†

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ABSTRACT: Medium-resolution hydrogen exchange methods have been used to examine the solvent accessibility of seven peptides in the regulatory subunit (r_2) of *Escherichia coli* aspartate transcarbamylase in the presence and absence of ATP and CTP. Both nucleotides are allosteric effectors of the holoenzyme; binding of ATP increases the affinity of the holoenzyme for the substrate L-Asp, while CTP has the opposite effect. Following Rosa and Richards (1979, 1981, 1982) and Englander et al. (1983, 1985), exchange-out curves for individual peptides were generated by adjusting the pH to 2.7 to quench exchange-out, digesting the protein with pepsin, separating peptides by reverse-phase HPLC, determining their radioactivity, and correcting for radioactivity lost during the analysis. Sixteen peptides from segments 1–11 and 76–153 were identified by amino acid and N-terminal analysis. Nine fell in regions where background was too high or were present at too low concentrations for exchange to be monitored. The number of protons whose exchange could be followed in peptides 1–11, 76–91, 78–90, 84–101, 93–112, 108–114, and 115–125 ranged from ≈1 (1–11, 108–114) to 10 (84–101) and 11 (93–112). The pattern of results obtained suggests that the structure of r_2 in solution is similar to that of the regulatory subunits in crystalline ATCase. Both CTP and ATP reduce rates of exchange from all seven peptides except 115–125. Although CTP slows exchange more than ATP, the effect is small except for peptides 76–91 and 78–90 which are near the nucleotide binding site. Since the seven peptides examined are distributed throughout r_2, these results provide direct evidence that the effects of nucleotide binding are propagated through the structure from the nucleotide binding site to the interchain interface between c and r chains. The fact that the results obtained for CTP and ATP are qualitatively similar and differ only in magnitude suggests that the structural basis of the opposing effects of CTP and ATP upon function is subtle.

Aspartate transcarbamylase (c_6r_6)[1] from *Escherichia coli* is a large (310 kilodalton) protein that binds substrates cooperatively and is allosterically regulated by ATP, an activator, and CTP, an inhibitor [for recent reviews, see Kantrowitz et al. (1980a,b), Kantrowitz and Lipscomb (1988), and Allewell (1989)]. The enzyme is comprised of two catalytic trimers (c_3) and three regulatory dimers (r_2) (Gerhart & Schachman, 1965; Weber, 1968) related by D_3 symmetry (Wiley & Lipscomb, 1968). Crystal structures of the unliganded enzyme (Honzatko et al., 1982; Ke et al., 1984) and its complexes with CTP (Kim et al., 1987) and the bisubstrate analogue *N*-phosphonoacetyl-L-Asp (PALA) (Krause et al., 1987) have been determined.

Much less is known about the mechanisms by which ATP and CTP regulate substrate affinity than about the mechanism of cooperative substrate binding. Both nucleotides bind in the anti conformation at the same site on the r chain (Honzatko & Lipscomb, 1982a,b; Banerjee et al., 1985), as well as at the active site (Tondre & Hammes, 1974; Suter & Rosenbusch, 1977). London and Schmidt (1972) have suggested that their opposing effects on function result from the difference in the positions of their six-membered heterocyclic rings relative to the protein surface. While the structural changes associated with substrate binding are large and readily related to function, those resulting from the binding of CTP are more subtle and influenced in the crystal by lattice interactions (Kim et al., 1987); those resulting from binding of ATP have not been analyzed. Although the effects of CTP and ATP upon several molecular parameters are consistent with a two-state Monod–Wyman–Changeux model (Howlett et al., 1977; Foote & Schachman, 1985; Newell et al., 1989), several types of evidence suggest that ATP, CTP, and substrates may act by different mechanisms. This evidence, derived from binding studies; experiments with "reporter groups", mutant enzymes, and substrate analogues; analyses of the pH dependence of the enzyme-catalyzed reaction; small-angle X-ray scattering; and analyses of equilibrium isotope exchange kinetics, has been summarized by Thiry and Hervé (1978), Hsuanyu and Wedler (1988), and Allewell (1989).

ATP and CTP produce large changes in the kinetics of hydrogen exchange from r_2 (Lennick & Allewell, 1981; Chan, personal communication). Hydrogen exchange methods that allow the exchange behavior of individual protons or groups of protons to be studied can therefore be used to probe the effects of nucleotide binding on various regions of the structure. The size of r_2 and the absence of a crystal structure preclude the use of ^{1}H NMR spectroscopy and neutron diffraction, respectively, to monitor individual protons; however, the medium-resolution fragment separation method developed by Rosa and Richards (1979, 1981, 1982) and Englander and colleagues (Englander et al., 1983, 1985; Ray & Englander,

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[1] Abbreviations: c_6r_6, native enzyme; c_3, catalytic subunit; r_2, regulatory subunit; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; OPA, *o*-phthaldialdehyde; PALA, *N*-phosphonoacetyl-L-Asp.

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1986; Louie et al., 1988a,b) enables net exchange rates from peptides within the native structure to be examined. This method takes advantage of the fact that the half-time for exchange at pH 2.7, 0 °C, is greater than 1.5 h. It is therefore possible to quench exchange by shifting to these conditions, digest the protein with an acid protease, separate the fragments by HPLC, and determine the amount of ^3H remaining with the peptide. When the raw data have been corrected for label lost during the analysis, the amount of ^3H associated with the peptide in the native protein at the time that exchange was quenched can be determined with a high degree of precision (Englander et al., 1985).

In this initial study, we have used this method to define the hydrogen exchange kinetics of several peptides in r_2 in the absence of nucleotides and to compare the changes in the kinetics of exchange from these peptides that result from binding CTP and ATP. Results obtained in the absence of nucleotides provide new information about the structure of r_2 in solution, while the experiments with CTP, an inhibitor, and ATP, an activator, allow the structural changes induced by these ligands to be compared. A preliminary report of the results obtained in the presence and absence of ATP has appeared (Burz & Allewell, 1986).

Materials and Methods

Proteins. c_6r_6 was prepared as previously described (Gerhart & Holoubek, 1967; Allewell et al., 1975) from the derepressed diploid strain developed by Gerhart and Holoubek (1967) and provided by G. O'Donovan or J. Wild (Texas A&M, College Station, TX) or from the transformed overproducing strain EK1104/pEK2 developed by Nowlan and Kantrowitz (1985) and provided by E. R. Kantrowitz (Boston College, Chestnut Hill, MA). Fermentations of the derepressed diploid strain were kindly performed by Weldon Crowe at Oak Ridge National Laboratories, Oak Ridge, TN. Purity was assessed by nondenaturing PAGE (Davis, 1964) and by pH-stat activity assays at pH 8.3 and was similar to previous preparations. c_6r_6 was stored as a precipitate in 3.6 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl, 0.2 mM EDTA, and 0.2 mM DTT, pH 8.3 at 4 °C.

r_2 was prepared following Yang et al. (1978) with the modifications introduced by Burz and Allewell (1982). r_2 was stored at 4 °C under 0.1 M Tris-HCl (pH 8.3), 3.6 M $(NH_4)_2SO_4$, 0.1 mM DTT, 2 mM $Zn(OAc)_2$, 180 μM benzamidine hydrochloride, and 50 μM streptomycin sulfate (to inhibit protelysis and bacterial growth, respectively) and used within 2 weeks of preparation. Purity was examined by microzone electrophoresis. Concentrations were determined by assuming an extinction coefficient of 0.32 mg·mL^{-1} (Blackburn & Schachman, 1977).

Hydrogen Exchange Experiments. r_2 was dissolved in 50 mM NaH_2PO_4–Na_2HPO_4 and 0.1 mM DTT, pH 7, and dialyzed overnight. Any undissolved protein was removed by centrifugation in an airfuge for 1–2 min. Final concentrations were 8–14 mg·mL^{-1}. ^3HOH (New England Nuclear, Boston, MA; 1 Ci·mL^{-1}) was added to give a final specific activity of 25–45 mCi·mL^{-1}, and exchange-in was allowed to proceed for 20–24 h at room temperature.

To initiate exchange-out, solid sucrose was added to a final concentration of 3%, and the solution was cooled to 0 °C and passed through a 1 × 8 cm Sephadex G-25 column equilibrated with the buffer and 2.5 mM ATP or CTP, if required. At suitable times, 0.3–0.4-mL aliquots were withdrawn, brought to pH 2.7 by the addition of a predetermined amount of 1 M H_3PO_4, and made 3% in sucrose. This solution was passed through a 1 × 6 cm Sephadex G-25 column equilibrated with 100 mM H_3PO_4–NaH_2PO_4, pH 2.7, and 2.5 mM ATP or CTP when appropriate, and 500–600-μL samples were collected. Twenty microliters of porcine pepsin (Worthington Diagnostics Systems, Inc., Freehold, NJ; 10–12 mg·mL^{-1} in 50 mM NaOAc) was added to these samples, and digestion was allowed to proceed for 5 min at 0 °C.[2] The samples were then applied to a Waters μBondapak C-18 reverse-phase column (Waters Associates, Inc., Milford, MA) packed in ice and equilibrated with 50 mM NaH_2PO_4, pH 2.8 (solution A), and eluted with a MeCN–NaH_2PO_4 gradient as follows: 100% solution A, 5 min; 7.5–15% (linear gradient) solution B (14% MeCN–86% NaH_2PO_4, pH 2.7), 2 min; 15% solution B, 5 min; 15–45% (linear gradient) solution B, 100 min. The elution profile was monitored at 230 nm with a Gilson Holochrome UV–visible detector, and a number of fractions of each peak were collected. Fractions were also collected in regions where the OD_{230} was low to assess the base-line radioactivity. The OD_{230} of each fraction was determined, and a 200-μL aliquot in 6 mL of Dioxint (National Diagnostics, Freehold, NJ) was counted for at least 10 min in an Intertechnique SL 32 liquid scintillation counter.

Peptide Identification. Peptides were identified primarily by amino acid composition. After HPLC solvents had been removed by lyophilization, hydrolyses were performed in sealed ampules under reduced pressure (<25 μm) in >100 μL of 4 N CH_3SO_3H + 0.2% 3-(2-ethylamino)indole at 110 °C for 24 h (Simpson et al., 1976). The solvent was then partially neutralized by addition of 100 μL of 3.5 M NaOH and removed by lyophilization. Precolumn OPA derivatization was carried out following Hill et al. (1979). Separation was carried out on a Rainin C-18 column (Rainin Associates, Woburn, MA) with a linear 10–40% acetonitrile gradient in 15 mM NaH_2PO_4 (Hill et al., 1982).

Identification was confirmed in some cases by N-terminal analysis of dansylated peptides following Gray (1972). The HPLC elution protocol was the same as for OPA–amino acids.

Extinction coefficients were calculated by assuming a value of 530 M^{-1}·cm^{-1} for the peptide bond (Englander et al., 1979) and contributions from side-chain chromophores of 65 (Cys), 665 (His), 165 (Met), 0 (Phe), 7500 (Trp), and 5000 (Tyr) (Wetlaufer, 1962).

Data Analysis. Corrections for ^3H loss during digestion with pepsin and HPLC separation were computed following Englander et al. (1985), taking into account effects on rates of exchange of both neighboring side chains (Molday et al., 1972) and organic chromatographic solvents, which reduce the rate of exchange exponentially (Englander et al., 1985). With the data and equations of Englander et al. (1985), it can be shown that under the elution conditions used here the relationship between t, the time that the peptide elutes after initiation of the 15–45% gradient, and t_{cor}, the time during which the same amount of exchange would occur in aqueous solvents, is given by

$$t_{cor} = 19.8 + (1 - e^{-0.011t})/(1.75)(0.011)$$

The first term corresponds to the summed contributions of the 15.4 min spent in aqueous solvent, the 2 min required to raise the concentration of solution B in the gradient from 0% to 15% (t_{cor} = 1.5 min), and the 5 min in 15% solution B (t_{cor} = 2.9 min). The second term corresponds to time spent in the 15–45% gradient; 1.75 is the slowing factor, defined by Englander et al. (1985) for the 15% solution, and 0.011 is a

[2] Autolytic fragments in the pepsin were removed by dialysis at a concentration of 10–12 mg·mL^{-1} against two changes of 50 mM NaOAc, pH 5.5, at 0 °C for 20 h. The solution was then titrated to pH 5.0 with 0.5 M NaOAc and stored in 50-μL aliquots in sealed ampules at −20 °C.

Table I: Peptides Identified by Amino Acid Composition

peptide	residues	peptide	residues
6b	1–10	17	84(85)–101
8	1–11	22b	90–106(107)
9	1–11 + contaminants	16	93–112
2b	2–7	20b	97–106 + contaminants
4b	2–10 + contaminants	4	108–114
10	78–89(91)	12	114(115)–125
13	76–91	10a	127–138
5b	84–90	19	128–153

constant [ln [(slowing factor)$_t$/(slowing factor)$_{t=0}$]/t].

^{3}H lost during analysis is given by

$$^3H_{loss} = \sum_{i=1}^{n-1}(1 - e^{-k_i t_{cor}})$$

where the summation is over $n - 1$ peptide protons and k_i is given by

$$k_i = (5.8 \times 10^{-5})(10^{k_a} + 10^{k_b})60$$

(Molday et al., 1972). k_a and k_b, the contributions that neighboring side chains make to the rates of acid- and base-catalyzed exchange, respectively, are given by

$$k_a = 3.0 - \text{pH} + r_a(i) + l_a(i + 1)$$

$$k_b = \text{pH} - 3.0 + r_b(i) + l_b(i + 1)$$

where 3.0 is the minimum in the curve of exchange rate versus pH, pH is the pH at which the peptides were separated, r_a and r_b reflect the contributions of side-chain i to acid- and base-catalyzed exchange of the peptide bond to the right in the sequence, and l_a and l_b are the corresponding factors for the next side chain (Molday et al., 1972). Calculations were performed with the FORTRAN program MEDRES written in this laboratory. Loss factors, $L = (n - 1)/(n - 1 - {}^3H_{loss})$, ranged from 1.36 (peptide 4) to 1.78 (peptide 12).

H_{rem} is then given by

$$H_{rem} = (111E_{230}/1.19C_0)(C/\text{OD}_{230})L$$

where C_0 and C are corrected counts in equal aliquots of the exchange-in solvent and an eluted sample and L is the loss factor (Englander et al., 1985). Curves of H_{rem} versus time were fit to the sum of two exponentials by nonlinear least-squares analysis with the program NONLIN written by Dr. Michael Johnson (Department of Pharmacology, University of Virginia, Charlottesville, VA).

Results

Figure 1 shows the results of a typical experiment in the absence of nucleotides. Sixteen peptides were identified by amino acid composition (Table I). In several cases, the position of peptide bond cleavage was confirmed by identification of peptides following each other in the sequence. Only one major peptide (15) could not be identified; it fell in a region of the profile that changed in the presence of nucleotides. The elution profile in the region of peptides 20b and 22b was also variable, even in the absence of nucleotides. Several fractions showed evidence of contamination by unrelated peptides; the fractional amino acid compositions of others (10, 13, 17, and 22b) indicated heterogeneous N- and C-termini. Identities of several peptides were confirmed by N-terminal analysis.

Background radioactivity (bars without hatches) is substantial particularly in the early part of the elution profile, probably as a result of ^{3}H leaching from precipitated protein adhering to the top of the column (S. W. Englander, personal communication). Some samples may also contain small

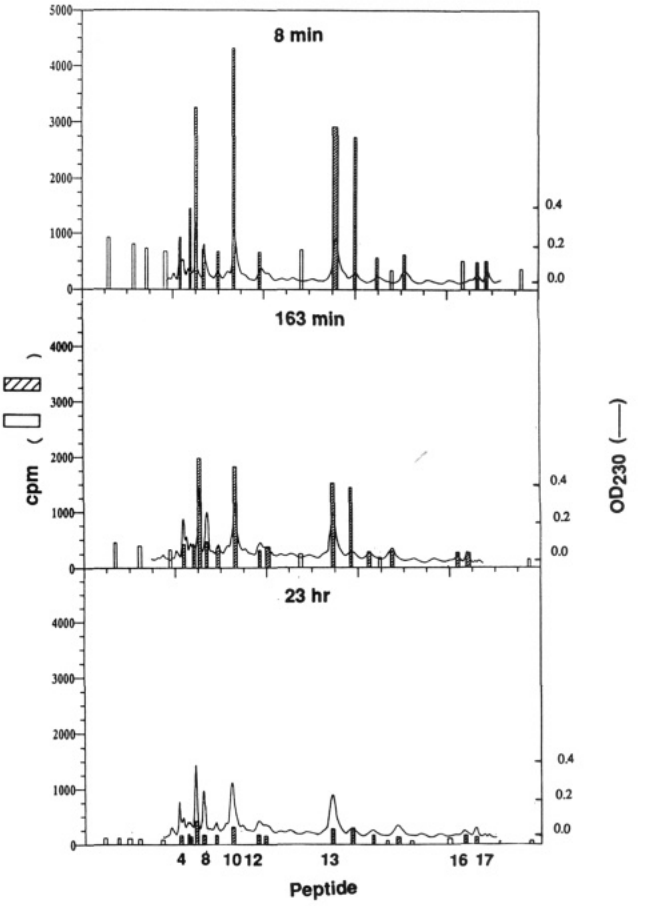

FIGURE 1: Elution profiles of r_2 at various times after initiating exchange-out in the absence of nucleotides. Bars without hatching indicate samples taken to assess background radioactivity. The protein was equilibrated with 25 mCi·mL^{-1} ^3H_2O in 50 mM Na_2HPO_4–NaH_2PO_4 and 0.1 mM DTT, pH 7, at room temperature for 22 h. Exchange-out was carried out in the same buffer at 0 °C.

amounts of peptides. Background corrections were calculated by drawing a smooth curve through the background peaks. Exchange from peptides eluting before peptide 8 could not be monitored because of the high background.

Amino acid compositions of the seven major peaks whose exchange could be monitored in both the absence and presence of nucleotides are given in Table II. Exchange-out curves for these peptides in the absence and presence of 2.5 mM CTP and ATP are shown in Figure 2. The results are similar to those of Burz and Allewell (1986) although H_{rem} values are larger. The similarity of the results for peptides 76–91 and 78–90 is indicative of the precision of the method. The error both within and between experiments was typically <0.1 proton. Curves through the points were derived by nonlinear least-squares analysis. All of the data could be fit by assuming only two kinetic classes. Except in the case of peptides 10 (78–90) and 13 (76–91) in the presence of CTP and ATP, >80% of the protons fell in the rapidly exchanging class. Rate constants ranged from 0.002 to 29 h^{-1}.

The location of these peptides in the three-dimensional structure of crystalline c_6r_6 is shown in Figure 3. The amino-terminal fragment 1–11 is close to the nucleotide binding site. Residues 1–7 are poorly defined on electron density maps, and residues 8–11 have no secondary structure, although the next residue, Ile-12, forms a hydrogen bond with the 4-amino group of CTP (Kim et al., 1987). 76–91 and 78–90 are also close to the nucleotide binding site; Tyr-89 also forms a hydrogen bond with the 4-amino group of CTP (Kim et al., 1987). 84–101 and 93–112 both contain residues that are part

Table II: Amino Acid Compositions of Peptides with cpm above Background[a]

	peak [residues]						
	8 [1–11]	13 [76–91]	10 [78–89(91)]	17 [84(85)–101]	16 [93–112]	4 [108–114]	12 [114(115)–125]
Ala	1.12 ± 0.2 (1)	2.04 ± 0.40 (2)	1.70 ± 0.74 (2)				0.97 ± 0.09 (1)
Arg		1.38 ± 0.05 (1)	1.07 ± 0.22 (1)	1.83 ± 0.25 (2)	1.8 (2)		
Asx	2.62 ± 0.45 (2)	4.36 ± 0.91 (3)	3.3 ± 0.3 (3)	2.54 ± 0.27 (2–3)	2.9 (3)	2.24 ± 0.38 (2)	
Cys[b]					nd (1)	+ (2)	nd (1)
Glx	2.41 ± 0.38 (2)	1.81 ± 0.74 (1–2)	1.40 ± 0.04 (1–2)[c]	2.22 ± 0.47 (2)	2.4 (1)		1.12 ± 0.10 (1)
Gly				0.95 ± 0.05 (1)	1.0 (1)		
His	1.04 ± 0.05 (1)						0.99 ± 0.02 (1)
Ile		1.49 ± 0.01 (1)	0.98 ± 0.42 (1)	1.30 ± 0.06 (1)	1.2 (1)		1.07 ± 0.13 (1)
Leu	0.99 ± 0.08 (1)	0.71 ± 0.3 (1)	0.18 ± 0.18 (0)	1.17 ± 0.17 (1)	1.8 (2)		
Lys[b]	+ (1)			+ (1)	nd (1)		
Met[b]	+ (1)						
Phe							1.04 ± 0.18 (1)
Pro[b]		nd (1)	nd (1)	nd (2)	nd (3)	0.91 (1)	1.25 (1)
Ser		0.65 ± 0.42 (0)		2.40 ± 0.47 (2)	2.5 (3)	1.02 ± 0.03 (1)	>3 (4)
Thr	1.14 ± 0.12 (1)	0.96 ± 0.04 (1)	0.95 ± 0.05 (1)				
Trp							
Tyr		1.88 ± 0.32 (1–2)[d]	0.84 ± 0.16 (1)	0.95 ± 0.33 (1)			
Val	1.24 ± 0.14 (1)	1.43 ± 0.51 (1–2)	1.42 ± 0.04 (1–2)[c]	2.02 ± 0.36 (2)	2.1 (2)	0.95 ± 0.40 (1)	1.06 ± 0.10 (1)

[a] The first number in each column is the experimental value with its associated error; the number in parentheses is the expected value. [b] Present, but could not be quantitatively determined with the procedure used. [c] Ambiguity due to variations in termini. [d] Sequence variable (Weber, 1968; Schachman et al., 1984; Kim et al., 1987).

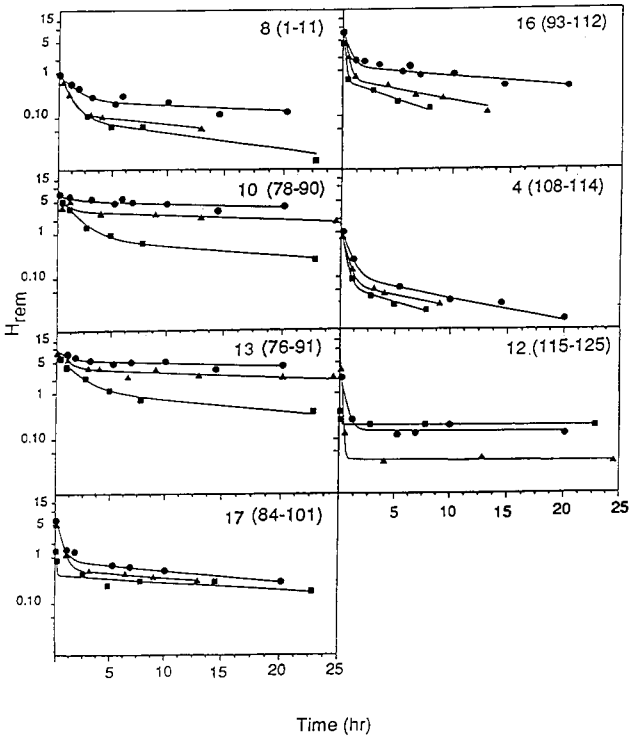

FIGURE 2: Semilog plots of H_{rem} as a function of time for individual peptides. (■) Control; (▲) 2.5 mM ATP; (●) 2.5 mM CTP in 50 mM Na_2HPO_4–NaH_2PO_4 and 0.1 mM DTT. Exchange-in was for 20–24 h at room temperature; exchange out was at 0 °C.

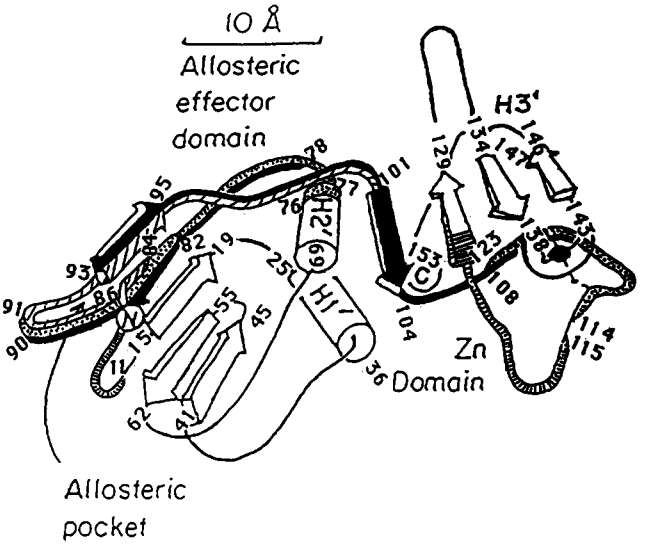

FIGURE 3: Diagram of the three-dimensional structure of the r chain in c_6r_6 [Honzatko et al. (1982), with the trace of S1′–S3′ and H1′–H2′ corrected as described by Kim et al. (1987)]. Peptides analyzed in this study are indicated by solid, hatched, or stippled regions.

of the binding site but also form the bridge which connects the nucleotide binding and zinc domains. 108–114 and 115–125 form a loop which encircles the Zn(II) ion at the interface between c and r chains. This region is critical to both c–r interactions and allosteric regulation (Cohlberg et al., 1972; Nelbach et al., 1972; Griffin et al., 1973); Cys-109 and Cys-114 are coordinated with the metal ion.

Although exchange-in is >90% complete under these conditions, many protons exchange too rapidly to be monitored. The number of protons whose exchange can be followed parallels the amount of β-structure in the structure of crystalline c_6r_6. Less than one proton remains unexchanged at the first time point in peptides 1–11 and 108–114, both of which lack secondary structure. In contrast, the exchange of 10–11 protons in peptides 84–101 and 93–112 can be followed; 5–6 residues in both peptides occur in β-structures in c_6r_6. Moles of ^3H associated with the remaining peptides (76–91, 78–90, and 115–125) at the first time point ranged from 3 to 6.

The difference in the results for 93–112 and 115–125 in the presence and absence of nucleotides is greater than experimental error and indicates that the effects of nucleotides propagate from the binding site to the c–r interface. Even at this level of resolution, however, the differences between ATP and CTP are quantitative rather than qualitative; both nucleotides reduce rates of exchange of all of the peptides examined, with the possible exception of 115–125, where both appear to produce a slight acceleration of exchange. The effects of nucleotides on individual peptides parallel their effects on net rates of exchange from intact r_2; both reduce the net rate of exchange, with CTP, which binds more tightly, having a greater effect than ATP (Lennick & Allewell, 1981).

The difference between ATP and CTP is greatest for peptides 10 and 13 (76–91 and 78–90). These peptides contain

residues that interact directly with CTP bound to crystalline c_6r_6 (Kim et al., 1987), and the differences in rates of exchange in the presence of CTP and ATP probably reflect differences in their modes of interaction with the binding site. Since the difference in the effects of CTP and ATP on the overlapping peptide, 84–101, is much less, the responsive protons in peptides 76–91 and 78–90 can be localized to residues 76–83.

A similar comparison between peptides 84–101 and 93–112 indicates that the responsive protons in segment 93–112 are located in peptide 102–112 in the Zn domain; although peptides 84–101 and 93–112 overlap, the effects of CTP and ATP on the rates of exchange from 84–101 are noticeably less than their effects on 93–112. In contrast to 76–91 and 78–90, however, the difference in the effects of ATP and CTP on exchange rates from 93–112 is small.

Discussion

The protons whose exchange has been examined in this study are among the more slowly exchanging. Although >90% of the exchangeable protons in r_2 are labeled under the conditions used for exchange-in (Burz and Allewell, unpublished results), only 40% exchange slowly enough for their exchange to be monitored by standard methods (Lennick & Allewell, 1981). This is to be expected since r_2 appears to be a relatively flexible structure; it is rather sensitive to proteolysis (McClintock & Markus, 1968), its melting temperature and enthalpy of thermal denaturation are low (Vickers et al., 1978; Edge et al., 1985), and assembly into c_6r_6 results in a tightening of the structure that is readily detectable by both hydrogen exchange measurements (Lennick & Allewell, 1981) and differential scanning calorimetry (Vickers et al., 1978; Edge et al., 1985, 1988).

In order to maximize the number of protons whose exchange could be monitored, a number of modifications were made in the procedure used previously (Burz & Allewell, 1986). Since errors in determining low OD_{220} values can be significant, the wavelength at which the elution profile was monitored was changed from 220 to 230 nm to reduce background, dialysis during exchange-out was eliminated to prevent the protein solution from gradually being diluted, and dioxane was eliminated from the eluent of the HPLC column to reduce background OD_{230}. Eliminating the dialysis step did not increase radioactive background, and eliminating dioxane had only a modest effect upon the elution profile. To prevent rapidly exchanging protons from being lost, samples to be analyzed were brought to pH 2.7 before being applied to the first pH 2.7 column and were not frozen and stored before analysis by HPLC. With these modifications, exchange of 36 of the 84 exchangeable protons in the 7 peptides examined could be monitored, compared to 12 in previous experiments. The fraction of exchangeable protons that are observable (40%) is comparable to that for the intact subunit under the same conditions (30% in the absence of nucleotides, 40% in their presence; Lennick & Allewell, 1981). The critical modification was adjusting the pH of the protein solution to pH 2.7 before applying it to the pH 2.7 column; apparently, rapidly exchanging protons were lost in the brief interval of equilibration to pH 2.7 on the column.

Very little is known about the structure of r_2 in solution and the extent that it differs from that of r_2 subunits in c_6r_6. The results obtained here indicate that the relative stabilities of various regions of r_2 parallel those of c_6r_6. Peptides 1–11 and 108–114 appear to be highly flexible, exchanging most of their protons too rapidly for exchange to be followed; neither of these peptides has any secondary structure in c_6r_6. All of the remaining peptides (76–91, 78–90, 84–101, 93–112, and 115–125) have some β-structure in c_6r_6, and all contain three or more protons whose exchange is slow enough to be monitored with this method. Peptides 84–101 and 93–112 have the largest number of slowly exchanging protons. For both of these peptides, the number of slowly exchanging protons is greater than the number of residues in the corresponding regions of c_6r_6 that form β-structure. It is possible that the structures of these regions of the molecule, which include the nucleotide binding site and the bridge between the allosteric and zinc binding domains, differ in r_2 and c_6r_6. Moreover, the reduction in net rates of exchange that results from incorporating r_2 into c_6r_6 (Lennick & Allewell, 1981) indicates that r_2 is more flexible when isolated than when incorporated into c_6r_6.

These results indicate that binding of CTP and ATP alters exchange rates of protons throughout r_2 and that the effects of nucleotides propagate from the binding site to the c–r interface. Although differences between ATP and CTP are greatest for protons in segment 76–83 near the nucleotide binding site, small quantitative differences in the exchange rates of protons in peptides 108–114 and 115–125 near the Zn(II) at the c–r interface can be demonstrated. The differences are, however, small, strongly suggesting that the opposing effects of CTP and ATP upon function depend upon subtle differences in structure.

These results parallel those of a ^{13}C NMR study in which the effects of CTP and ATP on the resonances of aromatic side chains of r_2 were monitored (Moore & Browne, 1980). Only 5 of the 12 reporter groups monitored were perturbed by nucleotide binding, and of these only 2 discriminated between CTP and ATP, strongly suggesting that the changes in tertiary structure resulting from nucleotide binding are minimal.

Both the hydrogen exchange and NMR results are also consistent with the crystal structures of c_6r_6 in the T (CTP-liganded) and R (PALA-liganded) states. The major differences in these structures arise from rigid body motions of domains. There are no changes in secondary structure and the only changes in tertiary structure in the r_2 subunit are in regions that were not examined in this study and that do not contain aromatic residues, segments 67–72 and 150–153 (Ke et al., 1989). The agreement between the crystallographic results and those for r_2 in solution suggests that the structures of r_2 in solution and in crystalline c_6r_6 are similar in both the absence and presence of nucleotides.

Studies of single-site mutants have provided many examples of systems in which major changes in biological function result from subtle changes in structure [cf. Alber and Matthew (1987) and Wells et al. (1987)]. Aspartate transcarbamylase provides an example of a naturally occurring protein whose biological function also appears to depend upon subtle conformational changes, both in the crystal and in solution.

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