Amide exchange rates in *Escherichia coli* acyl carrier protein: Correlation with protein structure and dynamics

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Abstract

The acyl carrier protein (ACP) of *Escherichia coli* is a 77-amino acid, highly negatively charged three-helix protein that plays a central role in fatty acid biosynthesis. Previous NMR studies have suggested the presence of multiple conformations and marginally stable secondary structural elements. The stability of these elements is now examined by monitoring amide exchange in apo-ACP using NMR-based methods. Because ACP exhibits many rapid exchange rates, application of traditional isotope exchange methods is difficult. In one approach, heteronuclear correlation experiments with pulsed field-gradient coherence selection have reduced the time needed to collect two-dimensional ^1H - ^{15}N correlation spectra to the point where measurement of exchange of amide protons for deuterium on the timescale of minutes can be made. In another approach, water proton selective inversion-exchange experiments were performed to estimate the exchange rates of protons exchanging on timescales of less than a second. Backbone amide protons in the region of helix II were found to exchange significantly more rapidly than those in helices I and III, consistent with earlier structural models suggesting a dynamic disruption of the second helix. Highly protected amides occur on faces of the helices that may pack into a hydrophobic core present in a partially disrupted state.

Keywords: fatty acid synthesis; heteronuclear NMR; ^{15}N labeling; protein dynamics

Acyl carrier proteins are small proteins of approximately 9 kDa that play a central role in fatty acid biosynthesis as the carriers of nascent fatty acid chains (Ohlrogge, 1987; Wakil, 1989). As such, they must interact with several different enzymes of the fatty acid synthetase complex and through these interactions have ample opportunity to influence the synthesis process. There is evidence that ACPs mediate recognition of certain intermediates in the biosynthetic pathway. In fact, changes in expression of ACPs or substitution of ACPs of different species can change fatty acid distributions in vivo (Simoni et al., 1967; Ohlrogge, 1987). Moreover, enzymes that use acyl-ACPs as substrates often show specificities not seen with less complex acyl analogues. For example, the specificity of the acyl-thioesterase activity of

cocoa plastids has been found to be significantly greater for acyl-ACPs compared to the corresponding CoA derivatives (Griffiths et al., 1993). Hence, there is considerable interest in the structural characteristics of ACPs.

The three-dimensional structure of *Escherichia coli* ACP, which is the only known structure of an ACP, has been determined by means of distance geometry, energy minimization, and simulated annealing algorithms using constraints derived from NMR NOE data (Holak et al., 1988a, 1988b). In line with earlier secondary structure predictions, the structure consists of a three-helix bundle. The structure determined using simulated annealing also contains a short fourth helix, as well as a structured turn before helix II. None of these structures, however, are of particularly high resolution. A detailed analysis of the NOE data reveals a pattern of conflicting constraints, and it has been suggested that conformational mobility is responsible for the limited success in attaining a high-resolution structure (Kim & Prestegard, 1989). A model for *E. coli* ACP consisting of two conformers in dynamic equilibrium also reduces the observed NOE conflicts and produces energetically more reasonable structures (Kim & Prestegard, 1989).

Comparison of the two structures postulated to be in dynamic equilibrium identifies regions that are likely to be highly mobile. On this basis, a great deal of disorder appears to exist in the loop

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Abbreviations: ACP, acyl carrier protein; CoA, Coenzyme A; DTT, dithiothreitol; FTIR, Fourier-transform infrared; HMQC, heteronuclear multiple-quantum correlation; HSQC, heteronuclear single-quantum correlation; IPTG, isopropyl β -D-thiogalactopyranoside; PMSF, phenylmethanesulfonylfluoride; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase incrementation.

regions of the protein. Helices tend to move as intact elements with the significant exception of helix II. In both the one-state model of Holak et al. (1988a) and in one of the two-state models of Kim and Prestegard (1989), this helix is disrupted, whereas the other two-state model contains a near-perfect helix, suggesting that helix II is relatively unstable.

Although the two-state analysis described above does provide a dynamic model consistent with the NOE constraint data, limitation to two states is arbitrary, and it is not at all certain that such specific models for conformational disruption are accurate. Thus, it is desirable to have independent, model-free evidence for the extent and localization of the mobility in *E. coli* ACP. The results of a recent FTIR spectroscopic study of *E. coli* ACP are consistent with a structure containing a combination of ordered and disordered helices (Ernst-Fonberg et al., 1993). Unfortunately, such methods are incapable of providing a correlation of dynamics with specific structural elements. More direct, site-specific information on the stability of secondary structural elements can be obtained by the measurement of amide exchange rates using NMR spectroscopy (Englander & Kallenbach, 1984; Englander & Mayne, 1992). Loss of intensity in resonances associated with amide protons is simply monitored as a function of time after dissolution of a fully protonated sample in D₂O. Modern 2D NMR methods and sequential assignment strategies allow adequate resolution and identification of amides by association of crosspeaks with specific sites in proteins of the size of ACP.

The rate of exchange of amide protons in native proteins is determined by a combination of their intrinsic exchange rate in the absence of secondary structure and the degree of protection from exchange due to the presence of secondary structure or solvent inaccessibility. To a reasonable approximation, the intrinsic rate is influenced by the chemical nature of the nearest-neighbor side chains and can be predicted based on knowledge of the primary structure and parameters available in the literature (Molday et al., 1972; Bai et al., 1993). In the limit where the lifetime of the "open," non-hydrogen bonded state is short compared to the intrinsic exchange rate (the EX₂ limit), the observed exchange rate k_{ex} is the product of the intrinsic rate k_{int} and an equilibrium constant for the opening/closing process P^{-1} (Hvint & Nielsen, 1966). This quantity may be thought of as a measure of the degree of protection of a given amide proton in a local-unfolding model for amide exchange (Englander et al., 1980; Englander & Kallenbach, 1984). Thus, the exchange rate of a given amide proton provides a direct measure of the presence of secondary structural elements and their stability.

In many proteins, the rates of amide exchange in structured elements can range from hours to days, and the time course of exchange is easily monitored. Accurate measurement of amide exchange rates in *E. coli* ACP, however, is made difficult by the lower level of structural stability and resulting rapid loss of amide proton signals in conventional hydrogen-deuterium exchange experiments. The use of gradient-selected heteronuclear multiple-quantum correlation methods with ¹⁵N-labeled protein allows the very rapid acquisition of 2D spectra, making possible the accurate determination of exchange on time scales down to minutes, while retaining adequate resolution for the association of rates with specific sites (Driscoll et al., 1990). We present in this paper measurements of amide exchange rates in *E. coli* apo-ACP as determined by hydrogen-deuterium exchange using HMQC spectra. We also present a new class of experiment that

can extend experimentally accessible time scales to even shorter times. These are based on experiments using water-selective magnetization transfer/HMQC spectroscopy (Kriwacki et al., 1993). The latter experiment replaces dissolution in a deuterated buffer with selective inversion of magnetization associated with water. This effectively labels water protons for a time on the order of water spin-lattice relaxation times. Because inversion can be repeated at will, labeling can be interleaved with 2D acquisition. Thus, this procedure can be used to monitor exchange on the 10-millisecond to second timescale.

Results

A prerequisite to measurement of specific amide exchange rates is assignment of amide proton and amide nitrogen crosspeaks. The basic strategy used follows that of Wüthrich (1986) for homonuclear systems. A scalar coupling experiment (TOCSY) was used to classify spin systems into a small number of residue types, whereas a cross-relaxation experiment (NOESY) was used to sequentially connect resonances through the appearance of H α /amide $i \rightarrow (i + 1)$ and amide/amide $i \rightarrow (i \pm 1)$ crosspeaks. Addition of an extra ¹⁵N dimension (TOCSY-HMQC and NOESY-HMQC) provided additional resolution and direct correlation with peaks in HMQC spectra to be used in exchange studies. The requisite ¹⁵N labeling of the protein was accomplished using an *E. coli* expression system developed by Hill et al. (1994). At high levels of expression, the production of properly processed holo-ACP, which contains the phosphopantetheine prosthetic group needed for charging with acyl chains, is low ($\approx 20\%$). However, previous NOE experiments have shown that in the absence of acylation, minimal interaction between the remote parts of the prosthetic group and the rest of the protein occurs (Holak et al., 1988a). Hence, we expect structural properties of the dominant apo form studied here to parallel those from the nondominant holo form studied previously.

Comparing strings of connected amino acid types with the known ACP sequence allowed unambiguous assignment of 73% of the apo-ACP sequence. Of these assignments, 89% were in agreement to within 0.10 ppm in chemical shift with previous holo-ACP assignments obtained in different buffer conditions (Holak & Prestegard, 1986). Given this high degree of agreement, most other assignments could be transferred from the earlier work. A superposition of key assignments on a 2D HMQC spectrum is shown in Figure 1A. Assignments are summarized in Table 1. Those assignments that differ from previous assignments are denoted with a dagger. In several cases these cluster around Ser 36 and may reflect the absence of the prosthetic group in this sample. NOE differences between the data collected here and previous studies are also minimal. Only NOEs to amide protons were detected in the 3D NOESY-HMQC experiment. Although these NOEs reflect primarily secondary structure and are not as likely to show evidence of small tertiary structural perturbations, an indication of the maintenance of similar secondary structures is all that is required to allow us to proceed with an investigation of amide exchange rates.

Amide exchange rates can be monitored by rapidly acquiring HMQC spectra, either after dissolution in D₂O, or after selective inversion of the water resonance. Figure 1B, C, and D shows the results of acquisitions after D₂O dissolution. A normal HMQC spectrum acquired in H₂O is shown in Figure 1A for comparison. It is clear that even at the earliest time point, only

Table 1. Summary of peak assignments for *E. coli* ACP^a

Residue	Amide H (ppm)	Amide N (ppm)	CαH (ppm)	Residue	Amide H (ppm)	Amide N (ppm)	CαH (ppm)
Ser 1	n/o ^b			Val 40	7.30	121.3	3.71
Thr 2	n/o			Glu 41*	8.06	113.0	3.72† (?)
Ile 3*	8.78	120.4	3.86	Leu 42	7.41†	122.2	3.74†
Glu 4*	8.72	117.6	3.74	Val 43	7.93	118.3	4.03†
Glu 5	7.90	116.6	3.92	MET 44	7.96	115.9	4.23†
Arg 6	8.46	119.3	4.27†	Ala 45*	8.33	121.6	4.29
Val 7	9.06	118.2	3.64	Leu 46*	8.47	120.1	4.30
Lys 8	8.30	116.1	3.84	Glu 47*	8.53	120.8	3.86†
Lys 9	8.21	120.1	4.11	Glu 48	7.91	115.9	3.88†
Ile 10	7.74	118.2	3.90	Glu 49	8.19	111.3	4.04
Ile 11	8.37	118.0	3.47	Phe 50	7.84	110.6	4.61
Gly 12	8.45	104.0	3.85†	Asp 51*	7.99	121.2	4.03†
Glu 13	8.26	119.1	3.48†	Thr 52	8.76†	111.0	4.30†
Gln 14	8.59	116.3	3.92	Glu 53	8.24	122.5	4.52
Leu 15	8.14	112.4	4.51	Ile 54	n/o		
Gly 16	7.80	109.2	3.98	Pro 55			4.53
Val 17	7.90	112.7	4.64	Asp 56	9.01	123.7	4.31
Lys 18	8.58	121.7	4.25	Glu 57	9.50†	115.8	4.11
Gln 19	8.87	121.5	3.89	Glu 58	7.23	115.2	4.35
Glu 20	9.50	115.8	4.15	Ala 59	8.56	122.9	3.67
Glu 21	7.89	115.9	4.32	Glu 60	7.64	113.2	4.00
Val 22	7.62	121.5	3.83	Lys 61	7.18	113.1	4.35
Thr 23	7.44	114.3	4.55	Ile 62	7.63	121.1	4.02
Asn 24	8.67	117.7	3.90	Thr 63	7.59	110.2	4.54
Asn 25	8.18	110.3	4.77	Thr 64	7.04	109.5	4.67†
Ala 26	7.44	122.2	4.23	Val 65	8.17	120.1	3.46
Ser 27*	10.07	116.2	3.90†	Gln 66	8.46	116.6	3.74
Phe 28	7.72	125.6	4.30	Ala 67	7.98	118.1	4.29
Val 29	8.82	116.2	4.22	Ala 68	7.88	120.2	4.07
Glu 30	8.41	115.5	4.07	Ile 69	8.11	117.4	3.25
Asp 31	7.92	112.3	5.11	Asp 70	9.26	117.5	3.74†
Leu 32	7.30	114.5	4.71	Tyr 71	8.25	120.1	4.13
Gly 33	7.47	105.4	3.94	Ile 72	8.20	119.7	3.60
Ala 34	8.60	122.2	4.22†	Asn 73	8.91	116.8	4.44
Asp 35	n/o			Gly 74	7.81	102.8	3.69
Ser 36*	8.39†	112.3	3.93† ^c	His 75	7.60	116.6	4.69
Leu 37	8.06	122.6	4.24	Gln 76	7.88	119.0	4.45
Asp 38*	8.52	120.8	n/o	Ala 77	8.15	130.0	4.21
Thr 39	8.13	111.0	3.71				

^a Residues assigned by comparison with previous assignments have been identified with an *, and assignments that show significant shift deviation from holo-ACP have been identified with a † (see Results).

^b n/o, Not observed in H₂O HMQC.

^c The peak at 3.93 ppm may be Hb.

a subset of resonances is observable. Following the loss of intensity in successive HMQC spectra (Fig. 1C, D), it is also clear that the resonances remaining lose intensity at variable rates.

Figure 2 shows a series of HMQC difference spectra taken with and without selective inversion of the water resonance (and peaks under the water resonance). All magnetization represented in these HMQC spectra must originate from the selectively excited resonances. In most cases this requires that magnetization reach amide sites by chemical exchange with water during the period τ . Hence, most of these peaks represent very rapidly exchanging amide sites. In this particular case, data acquired at pH 7.4 in the absence of Ca²⁺ are shown. However, spectra acquired in the presence of Ca²⁺ have similar crosspeak intensities and τ profiles. Note that the sites nearly complement those

shown in the first D₂O exchange spectrum (Fig. 1B). The sum of Figure 1B and Figure 2E is roughly equivalent to a normal HMQC spectrum (Fig. 1A). As τ is lengthened (Fig. 2B,C,D,E), it is clear that additional peaks arise and that those already present at 20 ms (Fig. 2A) increase in intensity. Hence, this experiment also offers the potential for quantifying exchange rates. It is applicable in a timescale similar to that reached by experiments based on transfer of magnetization after water saturation (Hoffman & Forsén, 1966) and is related to recent measurements of rapid exchange rates by Grzesiek and Bax (1993) and Gemecker et al. (1993) using doubly ¹³C- and ¹⁵N-labeled protein.

For the purposes of discussion, it is convenient to classify peaks observed in these HMQC spectra according to their qualitative behavior, rather than immediately attempt to extract rate

Fig. 1. Selected spectra from the amide $\text{H}_2\text{O}/\text{D}_2\text{O}$ exchange time-course. **A:** HMQC spectrum obtained in H_2O . **B, C, D:** Spectra obtained 9, 63, and 133 min after addition of D_2O (see the Materials and methods). Peak corresponding to Ser 27 (10.07, 116.2 ppm) is visible in the spectra corresponding to A and B but is not shown in the plot limits of this figure. This signal does not appear in the spectra of C and D.

constants for exchange. Amide protons in ACP can be classified into four different types based on their exchange properties as measured by H/D exchange and water-selective inversion/exchange. Type 1 amides are those that exchange slowly enough so that significant intensity is observable for the first several HMQC spectra of the H/D exchange experiment. For such crosspeaks, intensities can be fit to monoexponential or biexponential functions of exchange time:

$$I = \gamma + \alpha e^{-k_{\text{ex}} t} \quad (1A)$$

or

$$I = \gamma + \alpha_1 e^{-k_{\text{ex}1} t} + \alpha_2 e^{-k_{\text{ex}2} t}. \quad (1B)$$

Examples of the resulting fits are shown in Figure 3. Some biexponentiality of well-resolved resonances was seen, mostly in helix I. This may be due to chemical heterogeneity associated with incomplete processing of the methionine at the N-terminus of the protein.

Fig. 2. Selective inversion/exchange spectra showing the transfer of magnetization from protons at the chemical shift of water to amide protons. Note varying lengths of transfer time τ . **A:** $\tau = 20$ ms. **B:** $\tau = 40$ ms. **C:** $\tau = 60$ ms. **D:** $\tau = 80$ ms. **E:** $\tau = 100$ ms. Ser 27 (10.07, 116.2 ppm) is not visible in any of these spectra.

Type 2 amides exchange at a significantly faster rate than Type 1 amides. They are characterized by having crosspeaks in a normal H_2O HMQC, but not having crosspeaks in either a water-selective inversion experiment or the first point in a deuterium exchange experiment. These must exchange too slowly to give a water-selective exchange peak but too quickly to be retained 9 min after dissolution in D_2O . Using the ratio of the largest to smallest observed peak in the first spectrum after D_2O dissolution, one may estimate using Equation 1A that loss of a crosspeak would require a rate of at least 0.2 min^{-1} . Based on a quantitative analysis of the data obtained from the water-selective inversion experiment (see below), an amide not giving a crosspeak here would have to have a rate less than $\approx 60 \text{ min}^{-1}$. Thus, Type 2 amides correspond to those with exchange rates between 0.2 and 60 min^{-1} .

Type 3 amides will be those for which a crosspeak appears in the selective inversion experiment due to water exchange. These include the most rapidly exchanging amides. A crosspeak in the selective inversion/exchange experiment can unfortunately arise

Fig. 3. Examples of signal loss due to D_2O exchange as a function of time after addition of D_2O for three selected residues. Peak height is in arbitrary units.

not only from chemical exchange with water but also from NOE transfers from water, from α protons that happen to be chemical-shift degenerate with water, or from rapidly exchanging hydroxyl protons. Except for the case of tightly bound water molecules, NOE crosspeaks from water would be negative relative to positive autopeaks (Otting et al., 1991). No such negative peaks are observed. Also, water molecules that are tightly bound on the NMR timescale are not common and typically occur in proteins that are larger and have more stable secondary and tertiary structures than ACP (Gerothanassis, 1994). Hence, the possibility of direct NOE transfer from water is unlikely to be significant and for the semiquantitative analysis given here may be disregarded. The case of α -proton degeneracy is more common, and is more difficult to eliminate in the absence of ^{13}C -

labeled protein (Gemmecker et al., 1993; Grzesiek & Bax, 1993). However, crosspeaks arising in such cases can be identified using a combination of selective TOCSY/HMQC and ROESY/HMQC experiments (Kriwacki et al., 1993), or, even more simply, by inspection of the sequential assignments presented above. A few examples of crosspeaks arising from interaction of amide protons with intra- or ($n - 1$) inter-residue α protons are found. These have been eliminated from Type 3 classification and will be referred to as Type 2' based on their absence in the D_2O exchange spectra. It is also possible for cross-relaxation to occur between a hydroxyl proton in fast exchange with water and a nearby amide proton. This is most possible for three peaks observed in the selective inversion exchange experiment: Ile 3 (from Thr 2), Asn 24 (from Thr 23), and Glu 53 (from Thr 52). These have also been designated Type 2'.

Rate data and classification as to type are shown in Table 2. A summary is also presented graphically in Figure 4. Exchange rates for Type 1 amides are obtained using Equation 1, and protection factors derived from intrinsic rates using the parameters of Bai et al. (1993) are provided. For Type 1 amides, we are likely in the EX_2 limit, and interpretation in terms of protection factors is appropriate (Englander & Kallenbach, 1984).

Quantitative determination of exchange rates is also possible using the selective inversion exchange experiment. For many of the peaks appearing in these spectra, however, the signal/noise ratio is not adequate to warrant a complete analysis. Instead, we have chosen four amide protons (Ile 3, Glu 4, Gln 19, and Asp 56) that appear as well-resolved and high-intensity peaks to demonstrate the quantitation of exchange rates. It is possible to fit the intensity buildup profile using a function of the form

$$I = A[1 - \exp(-k\tau)], \quad (2)$$

where A is a scaling factor determined independently for each buildup curve (Gemmecker et al., 1993). This form neglects re-

Fig. 4. Amide exchange rate profile for apo-ACP (k_{ex} is given in units of min^{-1}). Secondary structural elements as defined by Holak et al. (1988a, 1988b) are indicated with rectangles. Amides for which rates could only be estimated are indicated with asterisks, and the bar length corresponds to the minimum rate consistent with the observed data (0.2 min^{-1} for Type 2 and 2' amides, and 100 min^{-1} for Type 3 amides). Missing bars indicate severe spectral overlap or amides not observed in the H_2O HMQC.

Table 2. Amide proton exchange rate constants and protection factors^a

Residue	Type	log k_{ex}	log P	Residue	Type	log k_{ex}	log P
Ser 1	ua			Val 40	1	-1.314 ± 0.009	3.4
Thr 2	ua			Glu 41	2	≥ 0.70	
Ile 3	2'	≥ 0.70		Leu 42	1	-1.04 ± 0.03	2.6
Glu 4	3	$\gg 0.70$		Val 43	1	-1.787 ± 0.006	3.3
Glu 5	2	≥ 0.70		MET 44	1	-1.641 ± 0.006	3.3
Arg 6	1	$-1.166 \pm 0.006^*$	3.1	Ala 45	2	≥ 0.70	
Val 7	1	$-2.05 \pm 0.02^*$	4.3	Leu 46	2	≥ 0.70	
Lys 8	1	$-1.79 \pm 0.02^*$	3.5	Glu 47	1	-2.412 ± 0.009	4.0
Lys 9	ol			Glu 48	1	$-1.93 \pm 0.02^*$	3.5
Ile 10	1	$-2.05 \pm 0.02^*$	4.1	Glu 49	1	-1.938 ± 0.004	3.5
Ile 11	1	-2.344 ± 0.004	3.7	Phe 50	1	-2.336 ± 0.007	4.2
Gly 12	1	$-1.51 \pm 0.02^*$	3.2	Asp 51	2	≥ 0.70	
Glu 13	1	-2.161 ± 0.007	4.5	Thr 52	2	≥ 0.70	
Gln 14	1	$-1.45 \pm 0.02^*$	3.4	Glu 53	2'	≥ 0.70	
Leu 15	1	$-1.62 \pm 0.02^*$	3.7	Ile 54	ua		
Gly 16	2	≥ 0.70		Pro 55			
Val 17	1	-1.345 ± 0.009	3.7	Asp 56	3	$\gg 0.70$	
Lys 18	3	$\gg 0.70$		Glu 57	ol		
Gln 19	3	$\gg 0.70$		Glu 58	1	-1.06 ± 0.02	2.7
Glu 20	ol			Ala 59	1	-1.320 ± 0.003	3.1
Glu 21	3	$\gg 0.70$		Glu 60	1	-1.00 ± 0.03	3.1
Val 22	ol			Lys 61	1	-1.10 ± 0.02	3.0
Thr 23	1	-1.04 ± 0.04	2.8	Ile 62	ol		
Asn 24	2'	≥ 0.70		Thr 63	2'	≥ 0.70	
Asn 25	ol			Thr 64	1	-1.274 ± 0.007	3.7
Ala 26	1	-1.09 ± 0.01	3.9	Val 65	2'	≥ 0.70	
Ser 27	1	-1.11 ± 0.02	3.7	Gln 66	1	-1.396 ± 0.006	3.2
Phe 28	1	-1.223 ± 0.006	3.9	Ala 67	1	-1.83 ± 0.02	4.2
Val 29	1	-1.09 ± 0.03	3.0	Ala 68	1	$-1.83 \pm 0.02^*$	4.1
Glu 30	3	$\gg 0.70$		Ile 69	1	-2.497 ± 0.007	4.5
Asp 31	1	-1.256 ± 0.007	2.8	Asp 70	1	-2.225 ± 0.005	3.6
Leu 32	1	-1.23 ± 0.03	3.0	Tyr 71	ol		
Gly 33	1	-1.08 ± 0.02	2.9	Ile 72	2	≥ 0.70	
Ala 34	1	-1.398 ± 0.006	3.9	Asn 73	1	-1.638 ± 0.003	3.4
Asp 35	ua			Gly 74	2	≥ 0.70	
Ser 36	2'	≥ 0.70		His 75	2'	≥ 0.70	
Leu 37	2'	≥ 0.70		Gln 76	2'	≥ 0.70	
Asp 38	2	≥ 0.70		Ala 77	3	$\gg 0.70$	
Thr 39	2	≥ 0.70					

^a Rates are given in units of min^{-1} . For residues that display biexponential kinetics (indicated with an * following the rate), only the rate constant corresponding to the largest value of α in Equation 1B is shown. ua, unassigned; ol, spectral overlap. See text for explanation of the numerical designations in the "Type" column.

laxation and incorporates a number of experimental variables into the constant A . The use of independent A values is not necessary, as the overall signal intensity should at a minimum be inversely correlated to the exchange rate k . A simple analysis allows us to explicitly include this correlation. For a chemical exchange crosspeak to be observed in this experiment, a transfer must occur from the water proton reservoir during the time τ , and that proton must remain on that amide site for the remainder of the experiment. It can be shown using arguments based on renewal theory (Appendix 1) that for a given t_1 point, the signal associated with a particular site will be given by

$$I = A_{\text{global}} \exp(-kt_H) [1 - \exp(-k\tau)], \quad (3)$$

where t_H is the length of the period required to create and rephase multiple quantum coherence ($1/J_{\text{NH}}$), and A_{global} is a

scaling factor for all buildup curves. This neglects any differences in the relaxation rates of the various coherences present during the exchange and coherence transfer periods. The normal dependence of I on t_2 and t_1 will result in the encoding of the exchange rate in the linewidths in the direct and indirect dimensions, respectively. A fit of experimental peak amplitudes using Equation 3 was carried out using a one-dimensional grid search to determine the value of A_{global} that minimizes the square of the residual after a nonlinear least-squares fit of the value k ($t_H = 15.5$ ms). The value of A_{global} for the four peaks in question was found to be 4.93 (arbitrary units) and the resulting exchange rates are given Table 3. The exchange rates are in good agreement with those predicted for unprotected amide protons from the equations of Bai et al. (1993).

We also attempted to fit additional peaks, for which direct cross-relaxation from α or hydroxyl protons is suspected. It is

Table 3. Exchange rates from fits of selective inversion exchange buildup curves

Residue	k (s^{-1}) from fit to Equation 4	Predicted rates for unprotected amides
Ile 3	18.6	30.9
Glu 4	6.2	8.1
Gln 19	22.4	13.4
Asp 56	12.4	13.5

interesting to note, for example, that the buildup curves of Asn 24 or Ser 36 could not be fit to a single value of A_{global} together with the four residues fit above. Attempts at fitting to a single A_{global} grossly underestimate the intensities of residues 24 and 36, presumably because Equation 3 does not take nonexchange contributions into account. The fact that Ile 3 can be fit to a single value of A_{global} along with data from residues that appear to have only exchange contributions supports the assertion that Ile 3 does not have a significant cross-relaxation contribution due to the hydroxyl group of Thr 2. We may also use Equation 3 to estimate the range of k that can be measured using $\tau \leq 100$ ms. Because the smallest peak observable has an intensity $I_{\text{min}} \approx 0.5$ on the arbitrary intensity scale used here, we may solve Equation 3 with $I = I_{\text{min}}$ using numerical methods to obtain an upper and lower limit for k of $\approx 150 s^{-1}$ and $\approx 1 s^{-1}$, respectively. Ideally, determination of exchange rates using selective inversion exchange would be done using an accordion-type experiment (Bodenhausen & Ernst, 1982; Kay & Prestegard, 1988), where it would be possible to deconvolute the effects of differential relaxation rates.

Discussion

The rate and protection factor data presented in Table 2 and Figure 4 can be used to qualitatively assess regions of structural stability in ACP. In general, the protection factors observed for Type 1 amides are significantly smaller than those that have been measured in other proteins, with most $\log P$ values significantly less than 4.5. There is a noticeable correlation with the sequential placement of most secondary structural elements in the published ACP structural models. The central part of helix I, postulated to run from Ile 3 to Gln 14, is recognizable as a region of relatively high protection factors ($\log P \approx 3.5$). The central portion of helix III, postulated in the region of Val 65 to His 75, is also recognizable as a region of protection similar to that of helix I. Helix IV (residues 56–63) and a structured loop (residues 26–34) are also recognizable as regions of somewhat lower protection factors ($\log P \approx 2.8$ – 3.5). Helix II, which had been postulated to run from Leu 37 to Asp 51, presents mixed results. Residues 40–44 have relatively high protection factors ($\log P \approx 2.8$), as does a region from 47 to 50 ($\log P \approx 3.5$ – 4.0). There is, however, a region of clear disruption with lower protection at residues 41 and 45–46. In addition to the previously identified helices, there is also a region of relatively high protection factors at residues 26–34. This is despite the fact that this region was identified as an unstructured loop in many of the structural models of ACP. The structural model derived using simulated annealing methods did, however, indicate a possible

structured turn in this location (Holak et al., 1988b). It is important to emphasize that these data were acquired using ACP in predominantly the apo form. It is not unreasonable to suspect that addition of the prosthetic group or acyl chain could alter the dynamics near the attachment point of the phosphopantetheine at Ser 36.

In order to better see correlations between protection factors and structures, it is useful to plot $\log k_{\text{obs}}$ versus $\log k_{\text{int}}$. In such a plot, amide protons with similar equilibria between open and closed forms will fall on a straight line of unit slope, with a y -intercept equal to $\log P'$, where P' is a representative protection factor for that group of amides (Fig. 5) (Englander & Kallenbach, 1984; Wagner, 1989). It should be emphasized that these lines are not intended to be least-squares fits but merely indicate groups of amide protons that have similar protection factors. Also, it does not necessarily imply that these residues are involved in the same unfolding transition.

For helix III, it is clear that the Type 1 amides fall into two groups, one consisting of the more protected residues 67, 68, and 69, and the other of the less protected residues 66, 70, and 73 (Fig. 5A). The former consists of residues near the middle and on the hydrophobic side of the amphipathic helix, whereas the latter are on the hydrophilic face (Fig. 6). This is reasonable from the point of view of a helix-fraying model, in which one would expect the inner residues of the helix to be the most protected. A higher degree of protection of the hydrophobic face is also not unexpected because the side chains of such residues may be involved in core packing, and amides may not freely exchange even when a secondary structural element is disrupted. Helix I shows similar behavior to helix III, having a more protected and a less protected group, with protection factors similar to those of helix III, except for the N-terminal end of the helix, which appears unprotected. Residues 7, 10, and 13 are again on one side of the helix, one that is very likely to be involved in hydrophobic packing. Note that residues 15 and 17, which previous structural studies have placed outside of helix I, have protection factors similar to those of the less protected group of the helix.

The behavior of helix II appears to be rather different, containing nearly unprotected amides at the N-terminal end, as well as a break in protection at residues 45 and 46. With the exception of residues 47, 48, and 50 at the C-terminal end, all of the Type 1 amides have protection factors comparable to those in the fast group of helix III (Fig. 5A,B). Even residues 47, 48, and 50, however, are less protected than the most protected residues of helices I and III ($\log P \approx 4.0$ versus 4.4). This suggests that most of helix II (with the possible exception of the region near Phe 50) is significantly less stable than helix III and is consistent with the suggestion of Kim and Prestegard (1989) that there may be serious disruption of the second helix. The higher degree of protection in the vicinity of Phe 50 may be indicative of more effective packing in this region of the protein. High protection here is also inconsistent with a simple helix-fraying model for amide exchange, suggesting that hydrophobic interactions may play a significant role in the behavior of this region of the protein.

A recurring motif in the above discussion is the presence of two groups of Type 1 amides, differing in their degree of protection, with the more highly protected consisting predominantly of nonpolar residues. A plot of all Type 1 amides using filled squares to denote polar and charged residues and open squares

Fig. 5. Plots of $\log k_{ex}$ versus $\log k_{int}$ for helix III (A) and helix II (B). Intrinsic rates were calculated from the parameters of Bai et al. (1993). Lines are of unit slope and indicate groups of residues with similar degrees of protection.

to denote nonpolar residues is shown in Figure 7. It is clear that polar residues consistently have a lower degree of protection, with the exception of glutamates 13 and 47, whereas the more highly protected group is dominated by nonpolar residues. This raises the possibility that even when a structural element is disrupted, an additional source of protection may persist. This type of behavior is not without precedent in the literature. The role of hydrophobic clusters in limiting amide exchange was proposed in a multistage, global-unfolding model of Wagner and Wüthrich (1979). The observation of two distinct groups of slowly exchanging amides has also been noted by Poulsen and co-workers in lysozyme (Pedersen et al., 1993; Thomsen & Poulsen, 1993). In their studies, "group 2" amides were located in a central core more than 4 Å from solvent and were characterized by larger protection factors and higher activation energies for exchange compared to "group 1" amides, which were located in a shell between the core and the surface. The energies of activation of these outer, less protected amides were found to be less than 50 kJ mol⁻¹, suggesting that breakage of few non-bonded interactions is needed to allow such amides to be exposed to solvent exchange (Thomsen & Poulsen, 1993). The buried core residues had significantly higher energies of activation, suggesting the presence of a larger number of restraining interactions. Even in cases of less complete disruption, additional

protection from putative packing effects has been noted, as in the case of calbindin D_{9k} (Skelton et al., 1992). It is interesting that such an effect is seen to such a degree in a relatively small and unstable protein such as ACP.

To summarize, evidence has been found for the existence of all four helices and a structured loop proposed to exist in *E. coli* ACP by previous modeling studies. In addition, this work supports the notion of *E. coli* ACP as an unusually mobile protein. This can be seen in the generally low values of protection factors, as well as in the presence of unprotected amides in regions that seem to show NOE connectivities characteristic of secondary structure. Of all of the secondary structural elements in *E. coli* ACP, helix II seems to have more than its fair share of mobility. Work currently in progress will study the degree to

Fig. 6. Schematic representation of helix III of *E. coli* ACP, showing its amphipathic character, as well as the correlation between hydrophobicity and degree of protection (see Discussion). Protection factor data are shown for Type 1 amides, and Type 2/2' amides are indicated with an asterisk. Data for tyr 71 could not be obtained due to spectral overlap.

Fig. 7. Plot of $\log k_{ex}$ versus $\log k_{int}$ for all Type 1 amides in apo-ACP. Nonpolar residues are indicated with open squares, and polar and charged residues are indicated with filled squares. Lower dashed line indicates a group of nonpolar residues throughout the protein that have similar degrees of protection. Upper dashed line indicates an approximate lower limit of protection for most polar and charged residues.

which dynamics are modulated by the addition of prosthetic group and acyl chains.

Materials and methods

Sample preparation

E. coli strain MgT7/pLysS cells transformed with a pET-3a vector containing the synthetic ACP gene under control of a T7 promoter were grown in MOPS minimal media at 30 °C with 1 g/L $^{15}\text{NH}_4\text{Cl}$ (99% enrichment) as the sole nitrogen source (Neidhardt et al., 1974). Cultures were induced with 0.6 mM IPTG at an OD_{600} of 1.0. Approximately 6 g of wet cells were harvested per liter of culture. ACP was purified using a method described in detail elsewhere (Hill et al., 1994). Briefly, cell lysis was achieved by resuspension of the cell pellet in 10 mM EDTA, 50 mM Tris buffer, pH 8.0, containing 0.1% PMSF, followed by ultrasonication until the suspension was fluid. The cell debris was pelleted and the supernatant adjusted to 60% ammonium sulfate at 4 °C. Following centrifugation, the high salt supernatant was precipitated at pH 3.5 and the ACP-containing pellet was reduced based on the method of Rock and Cronan (1980). After dialysis, the ACP solution was chromatographed on a Sepharose FF anion-exchange column (Sigma). Typical yields were on the order of 25 mg protein/L of culture. Analysis by HPLC and electrospray mass spectrometry showed some structural heterogeneity, corresponding in part to presence and absence of the N-terminal methionine and phosphopantetheine prosthetic group. Approximately 80% of the ACP overexpressed from this construct is in the apo form, with a free hydroxyl at serine 36. The dominance of this form and the minimal spectral perturbation produced by the other forms make it possible to conduct experiments on the anion-exchange purified product.

NMR samples were prepared in an H_2O buffer using 100 mM deuterated acetate, pH 6, and in some cases 10 mM calcium chloride. A small amount of DTT was added to prevent dimerization of the holo ACP during the course of the experiment. Final concentration of protein was estimated at 2 mM from signal strength in 1D NMR spectra. For the proton-deuterium exchange studies, the sample was lyophilized for the minimum time needed to remove most of the H_2O and minimize the loss of acetic acid under vacuum. The sample was then resuspended in D_2O containing 10 mM deuterated acetate buffer, pH 5.0, and immediately placed into a previously tuned and calibrated NMR spectrometer. All proton-deuterium exchange spectra were obtained in the presence of Ca^{2+} . After data collection, the pH was found to be 6.2 (uncorrected for deuterium isotope effect). Water-selective magnetization transfer/HMQC spectra were acquired in H_2O using 100 mM deuterated acetate buffer at pH 6.0 and 7.4, both in the presence and absence of 10 mM calcium chloride.

NMR spectroscopy

NMR spectra were obtained at a 500-MHz proton frequency using a GE Omega spectrometer equipped with an S-17 pulsed field-gradient accessory. All spectra were acquired at a temperature of 30 °C. Spectral data were processed using the program FELIX (versions 2.1 and 2.3, Biosym Technologies). Sequential assignment of amide proton, $\text{C}\alpha$ proton, and amide nitrogen chemical shifts relied on 3D TOCSY/HMQC and 3D NOESY/

HMQC spectra collected using sequences analogous to those described in earlier literature (Fesik & Zuiderweg, 1988; Marion et al., 1989a). For both the TOCSY and NOESY/HMQC, 512 complex points were acquired in the direct dimension (t_3), whereas 128 and 32 complex points were acquired in the indirect ^1H (t_1) and ^{15}N (t_2) dimensions, respectively. Quadrature in the indirect dimensions was achieved by States-TPPI in t_1 and gradient cycling in t_2 (Marion et al., 1989b; Tolman et al., 1992). Spectral widths of 5,988 Hz in f_1 and 2,273 Hz in f_2 were used. The spectral width of f_3 was 3,300 Hz for the NOESY/HMQC, and 3,623 Hz for the TOCSY/HMQC. Skewed sinebell apodization was used in all dimensions, and f_1 and f_2 were zero-filled to 256 and 64 complex points, respectively.

A gradient-selected HMQC experiment (Tolman et al., 1992) was used for measurement of H/D exchange rates, using 32 complex points in t_1 , 256 complex points in t_2 , and spectral widths of 2,273 Hz in t_1 and 3,603 Hz in t_2 . A total of 38 2D spectra were acquired consecutively over 17 h: 10 with 4, 8, and 16 transients/ t_1 increment, 6 with 32 transients/ t_1 increment, and 2 with 64 transients/ t_1 increment. The collection of 32 complex points with 4 transients/ t_1 increment required ≈ 6 min and data collection was initiated 6 min after addition of D_2O buffer. These times are moderately short but can be shortened further by the use of the enhanced version of the HSQC experiment introduced during the course of our data collection (Kay et al., 1992). All spectra were processed identically, with skewed sinebell apodization and zero-filled to 1,024 points in t_1 and 256 points with first point linear prediction in t_2 . Spectra acquired with more than four transients/ t_1 increment were normalized by the appropriate factor to allow direct comparison of signal intensities.

Amide proton signal decay as a function of time was followed by measurement of peak height as a function of the time at the intensity maximum of each peak in a 2D spectrum. Decay curves were fit using both one- and two-component exponentials using the program SplMod (Provencher & Vogel, 1983; Vogel, 1988). In addition to individually analyzing single sets of data corresponding to "time vectors" along the intensity maximum of each crosspeak, sets of nine "time vectors" corresponding to the intensity maximum and the eight vectors within one point of the maximum were analyzed together using the simultaneous fit capability of SplMod. All values and standard deviations of rate constants given below are those obtained from the nine-vector simultaneous fit.

Estimation of exchange rates on subsecond timescales were obtained using a water-selective 2D HMQC experiment that began with an off-resonance selective inversion of water protons using an amplitude-modulated low-power pulse shape developed by Shinnar and LeRoux (Shinnar et al., 1989; Pauly et al., 1991; John et al., 1992). Inversion was followed by a transfer time τ , and detection through a gradient-selected HMQC experiment (Kriwacki et al., 1993). The inversion pulse and receiver phase were cycled appropriately to produce a difference experiment. The gradient pulses used for multiple quantum coherence selection in the HMQC were also sufficient for water suppression. Gradient pulses were also used at the beginning and end of the mixing time to dephase residual transverse magnetization and reduce artifacts due to radiation damping. It was determined that water remains fully inverted for over 200 ms (data not shown). Mixing times of 20–100 ms and Shinnar–LeRoux pulse widths of 50 and 80 ms were used. Four dummy scans were used

to establish a steady state, and a delay of 1.8 s was used between scans. Sine-bell gradient pulses of 1 ms duration were applied along three orthogonal axes simultaneously (≈ 30 G/cm per axis). Total acquisition time per 2D data set was less than 8 h. Data set size, spectral widths, and processing parameters were the same as for the H/D exchange HMQC spectra above.

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Appendix 1

We derive here an expression for the signal intensity for a selective inversion exchange pulse sequence neglecting the effect of cross-relaxation. As stated in the text, a transfer must occur from the water proton reservoir during the time τ , and the proton must remain on that amide site for the remainder of the experiment. If we assume that exchange is ergodic and can be represented by a Poisson process, we may model this by following the stochastic path of a single proton undergoing exchange between the water reservoir (state 1) and an amide site (state 2). If the proton begins in state 1 at time $t = 0$, the probability of a state 1 to state 2 exchange event in the time interval $(t, t + dt)$ is given by

$$h_I(t) = \frac{k_1}{k_1 + k_2} [k_2 + k_1 e^{-(k_1 + k_2)t}], \quad (\text{A1})$$

where $1/k_1$ and $1/k_2$ are the mean lifetimes of a proton in states 1 and 2, respectively (Cox, 1962). The probability that no reversal of the exchange event occurs for an additional time t is simply

$$U_2(t) = \exp(-k_2 t). \quad (\text{A2})$$

Signal intensity I_0 is directly proportional to the probability of an irreversible exchange event at any time during the exchange time τ multiplied by the probability of no reversal during the time required to create and rephase multiple quantum coherence ($t_H = 2[1/2J_{NH}]$). During t_1 and t_2 evolution, any reverse exchange results only in broadening of lines, so integration eliminates the need for consideration of exchange events during these periods. Thus, in the absence of relaxation:

$$I_0 \propto U(t_H) \int_0^\tau h_I(t) U(\tau - t) dt \quad (\text{A3})$$

$$= \frac{k_1}{k_1 + k_2} e^{-k_2 t_H} [1 - e^{-(k_1 + k_2)\tau}]. \quad (\text{A4})$$

Now, $k_1 \ll k_2$ due to the vast excess of water, so the pre-exponential term reduces to an effective equilibrium constant k_1/k_2 , relating populations of the water and amide states, and the buildup is dominated by k_2 :

$$I_0 \propto \frac{k_1}{k_2} e^{-k_2 t_H} (1 - e^{-k_2 \tau}). \quad (\text{A5})$$

The ratio of populations k_1/k_2 is identical for all amide sites and can be included as a part of a global scaling factor A_{global} . In the case of the experimental data presented above, any exchange effects on the line-widths will be overwhelmed by the effect of apodization. Therefore we may, to a reasonable approximation, use peak amplitudes rather than peak volumes.

We neglect the effect of T_1 relaxation and/or radiation damping of water in the above discussion, because it was experimentally determined that under the conditions of our experiment water remains fully inverted for more than 200 ms. We may, however, evaluate the effect of T_1 and T_2 relaxation of amide protons following exchange. These effects are easily included in the limit where exchange rates and concentration factors prevent large perturbations to water spin-lattice relaxation. Amide relaxation simply becomes another way to lose magnetization from an amide site once it exists, so that k_2 in the factor $U_2(t)$ is replaced with $(k_2 + \rho_1)$ or $(k_2 + \rho_2)$, depending on whether T_1 or T_2 relaxation is active during the corresponding time period ($\rho_i = 1/T_i$). The resulting equations in the limit of $k_1 \ll k_2$ and $k_1 \ll \rho_1$ are

$$I \propto e^{-(k_2 + \rho_2)t_H} \int_0^\tau h_I(t) e^{-(k_2 + \rho_1)(\tau - t)} dt \quad (\text{A6})$$

$$= e^{-(k_2 + \rho_2)t_H} \frac{k_1}{k_2 + \rho_1} [1 - e^{-(\rho_1 + k_2)\tau}]. \quad (\text{A7})$$

It can be seen from Equation A7 that the effects of T_1 and T_2 relaxation are separable and that the magnitude of the effect of T_1 relaxation is dependent on τ , k_1 , and k_2 . The resulting error may be evaluated by considering the ratio I/I_0 . For T_2 relaxation alone, this error is $\exp(-\rho_2 t_H)$, which may be incorporated into A_{global} if ρ_2 is assumed to be constant for all amide sites. The error due to T_1 relaxation is most easily evaluated by numerical evaluation of I/I_0 for typical values of k_1 , k_2 , and ρ_1 ($\rho_2 = 0$) (Peng & Wagner, 1992). If we use the values $k_2 = 15 \text{ s}^{-1}$, $k_1/k_2 = 10^{-4}$, and $\rho_1 = 4 \text{ s}^{-1}$, we find that I/I_0 ranges from 0.96 ($\tau = 20 \text{ ms}$) to 0.86 ($\tau = 100 \text{ ms}$). This will lead to a slight underestimate of intensities at longer mixing times. It is clearly necessary to obtain site-specific estimates of ρ_1 and ρ_2 if highly accurate measurement of exchange rates are desired. For the semiquantitative analysis presented here, these effects are ignored and Equation A5 is used.