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Complex patterns formed by motile cells of *Escherichia coli*

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WHEN chemotactic strains of the bacterium *Escherichia coli* are inoculated on semi-solid agar containing mixtures of amino acids or sugars, the cells swarm outwards in a series of concentric rings: they respond to spatial gradients of attractants generated by uptake and catabolism[1-3]. Cells also drift up gradients generated artificially, for example by diffusion from the tip of a capillary tube[4] or by mixing[5]. Here we describe conditions under which cells aggregate in response to gradients of attractant which they excrete themselves. When cells are grown in semi-solid agar on intermediates of the tricarboxylic acid cycle, they form symmetrical arrays of spots or stripes that arise sequentially. When cells in a thin layer of liquid culture are exposed to these compounds, spots appear synchronously, more randomly arrayed. In either case, the patterns are stationary. The attractant is a chemical sensed by the aspartate receptor. Its excretion can be triggered by oxidative stress. As oxygen is limiting at high cell densities, aggregation might serve as a mechanism for collective defence.

Examples of patterns formed in semi-solid agar are shown in Fig. 1. The spots or stripes are dense accumulations of cells visualized by scattered light. They arise sequentially in the wake of a spreading circular band, as shown in Fig. 2. In this experiment, the band appeared at the edge of the inoculum after about 10 h and migrated outwards at a constant speed of 0.75 mm h^{-1}. Every few hours, new sets of stripes or spots appeared in its wake. These aggregates first appeared as faint inhomogeneities that rapidly intensified. While bright, they contained vigorously motile bacteria, as judged by phase-contrast microscopy. At this stage of development, the spots could move as integral structures as far as 1 mm, but usually they remained in one position. The accumulation was so dense that the cells appeared to sink to the bottom of the petri plate. Later on, the spots faded (scattered less light). At this stage, all the cells in the aggregate were nonmotile. Compare, for example, the intensities of the spots and stripes in the lower right-hand quadrant of the pattern of Fig. 2 at 28 and 32 h. By 32 h, another bright stripe and set of bright spots had been added; the older structures, while still compact, had faded. The final pattern is similar to that of Fig. 1*a* but less regular. Note the major defect in the lower right-hand quadrant comprising a series of abnormally long stripes or arcs subtending successively smaller angles. For a photograph of this pattern taken at about 47 h, see Fig. 3 of ref. 6. All the patterns shown in Figs 1 and 2 'bred true': they could be regenerated by inoculating a fresh plate with cells taken from any part of the original pattern.

Patterns of lower symmetry could be generated on a much shorter timescale (5 to 15 min) if cells were grown in a liquid medium on a single carbon source (as in Fig. 1, but without agar or vital dye) to a density of about 10^8 cells per ml, poured as a thin layer into an empty plate, and then exposed to intermediates of the tricarboxylic acid (TCA) cycle, such as succinate, fumarate, or malate (below). Spots formed near the top of the liquid and later settled onto the bottom of the plate (data not shown). If the medium was stirred any time within 9 min after the spots appeared, they reappeared; if it was stirred later, they did not. These patterns remained stable for about 25 min and then dispersed, fading away after about 30 to 40 min. They could not be resurrected by further addition of the same substrate, unless this addition was made 3 to 4 h later.

For patterns to arise on such a short timescale, active accumulation is required. Neither growth nor mutation can be involved, because the spots appeared in a fraction of a generation time. As spots also formed in agar, convection is not required. The most likely alternative is that the cells aggregate in response to a chemical signal generated by the cells themselves.

Cells of *E. coli* are attracted by oxygen and other electron acceptors, a variety of sugars, amino acids and peptides (reviewed by Macnab[7]), and salts[8]. To determine which sensory system might be involved, we tested a number of different strains both in semisolid agar and in liquid. Patterns formed only when cells were motile, chemotactic, and responsive to aspartate or its analogues. Cells that were generally nonchemotactic but tumbled as well as swam smoothly (for example *cheR cheB* cells) formed diffuse swarms[3] but failed to generate patterns. Cells that were nonmotile (*mot A, mot B*) or that only swam smoothly (*tsr, tar, trg* cells, or cells deleted for all cytoplasmic *che*-gene products) did neither. The most reproducible patterns were obtained with strains sensitive to aspartate but insensitive to serine (*tsr* strains).

Pattern formation was suppressed when substances sensed by the aspartate receptor were added to the medium. This suppression was nearly complete at concentrations comparable to the receptor–ligand dissociation constant (for example with L-aspartate at 5 μM, α-methyl-D,L-aspartate at 125 μM, or L-glutamate at 1 mM). Two experiments with the nonmetabolizable analogue α-methyl-D,L-aspartate[9] are shown in Fig. 3. The sequence observed with progressively higher concentrations of the inhibitor was from spots, to stripes of increasing separation, to loss of all structure. As the inhibitor was nonmetabolizable, this must be an effect of reduced chemotactic sensitivity, not altered growth.

In an attempt to learn more about the conditions that might be required for the generation of patterns in semisolid agar, we surveyed a variety of carbon sources: glycerol, pyruvate, acetate, amino acids, sugars and intermediates of the TCA cycle. Only the more highly oxidized intermediates of the TCA cycle worked well and then only at relatively high concentrations (for example succinate or fumarate at 2–5 mM, or malate at 6 mM, concentrations far higher than those required to saturate growth[10]). As evident in Fig. 1, reproducible changes in geometry occurred with moderate changes in the composition of the growth medium. For a given composition, however, the same geometry was produced with different strains, provided that the cells were sensitive to aspartate (data not shown). Availability of oxygen was also important. Patterns were more robust with thin layers of agar or liquid (less than 2 mm). They were not observed with thicker layers (more than 3 mm).

A similar survey was conducted with cells grown in liquid media. Spots appeared when cells were grown on any single carbon source (except aspartate), provided that the cell suspensions were exposed to intermediates of the TCA cycle (for example succinate, fumarate or malate at 5 mM). α-Ketoglutarate gave a weak response; oxaloacetate gave none. For these experiments, the sensitivity of the assay was enhanced by addition of viscous agents known to supress stirring and increase swimming speeds (for example, 0.2% (w/v) Methocel 90 HG, Dow; ref. 11).

These results suggest that excretion of aspartate or an aspartate analogue might be triggered by hazardous byproducts of respiration, such as peroxides or superoxides[12,13]. Indeed, when

cells were grown on intermediates of the TCA cycle (including α-ketoglutarate) and challenged by the addition of hydrogen peroxide (5 mM), inhomogeneities leading to spot formation appeared within a few seconds. This did not occur when cells were grown on the other carbon sources (including tryptone), with aspartate-blind mutants, or in the presence of α-methyl-D,L-aspartate. Whether other agents known to induce oxidative stress are effective remains to be determined.

A direct test for excretion of aspartate or an aspartate analogue was carried out with a chemotaxis assay in which cells migrate through a porous plate separating two identical stirred chambers[14]. A culture grown on α-ketoglutarate was divided into two parts. Cells were removed from one part by filtration, and the filtrate was used to fill the chemotaxis apparatus. Then cells from the other part were added to chamber 1 at a final concentration of 10^7 cells ml^{-1}. The density of cells in chamber 2 was determined from the amount of light scattered from the beam of a laser diode and recorded on a strip chart, as shown in Fig. 4. Cells added to chamber 1 at (a) diffused from chamber 1 into chamber 2, causing the density of cells in chamber 2 to increase with time. From the density of cells in each chamber and the slope of the curve between 18 and 32 min, the diffusion coefficient was estimated to be 4.8×10^{-6} cm^2 s^{-1}, as expected for cells with wild-type motility[14]. To test for excretion of an attractant, hydrogen peroxide was added to the chemotaxis apparatus. To avoid artefacts due to a peroxide gradient, the same amount was added to each chamber. For technical reasons (to prevent bulk flow through the porous plate), these additions had to be made separately. The first addition (at b) diluted the cells in chamber 2 by about 10%, transiently reducing the output

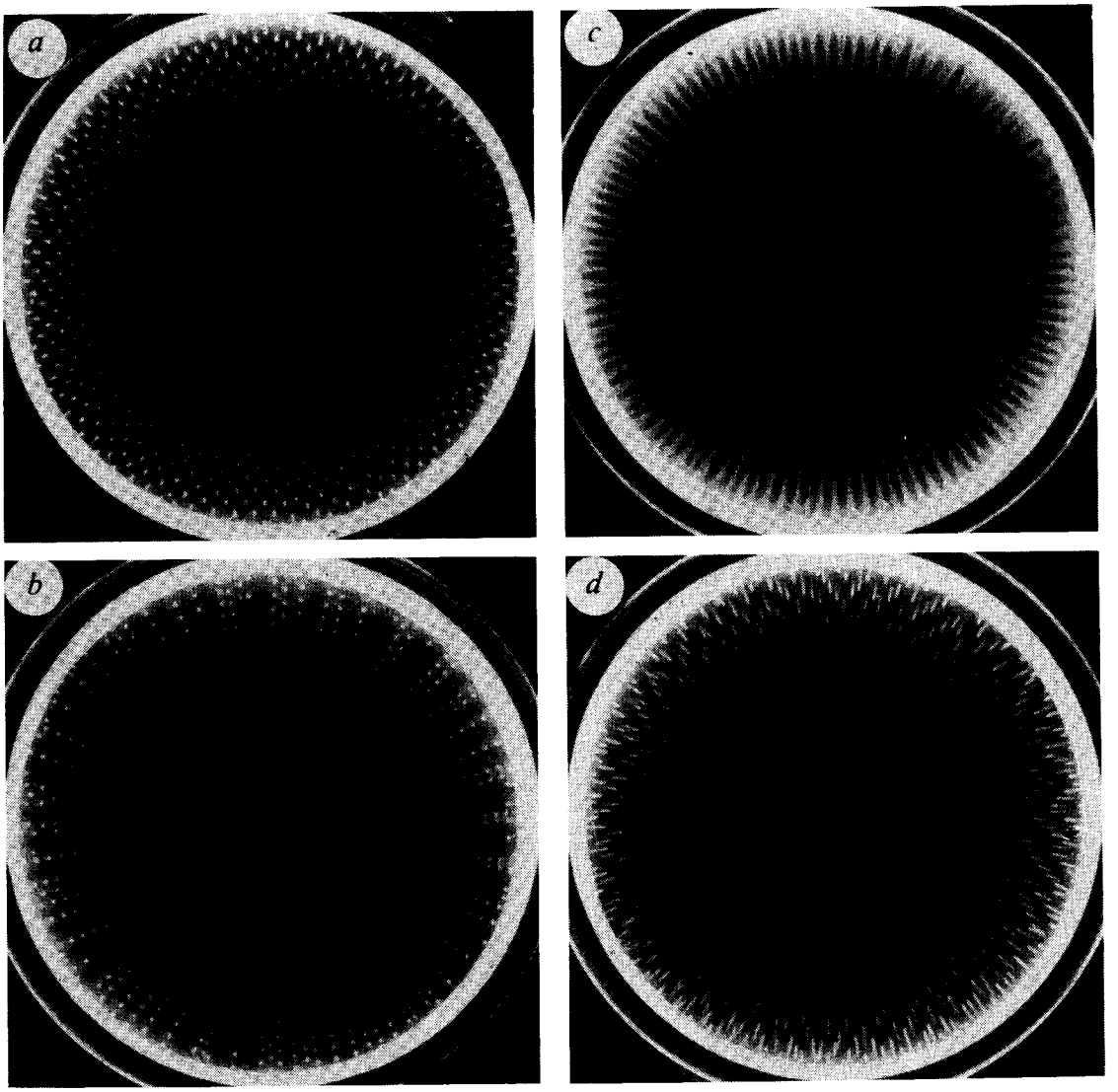

FIG. 1 Patterns formed by *E. coli* in semi-solid agar. The white ring near the edge of each plate is an artefact due to the mode of illumination. The inside diameter of this ring is 7.5 cm *a*, Sunflower-like arrays of spots. At smaller radii the spots tend to appear on circles, at larger radii on intersecting clockwise and counterclockwise spirals. *b*, Radial arrays of spots. *c*, Radial arrays of spots and stripes. *d*, Spots with radial tails arrayed in chevrons. METHODS. Cells were grown to saturation on M9 minimal medium[20] containing 20 μg ml^{-1} each of L-threonine, L-leucine, L-histidine and L-methionine (required for growth) and 5 mM α-ketoglutarate (a carbon source). This suspension (20 μl) was inoculated at the centre of an 8.5 cm internal diameter petri plate on 9.6 ml of a similar medium containing 0.22% agar (Difco Bacto-Agar), a different carbon source, and (when it was desirable to enhance image contrast) a vital dye (either tetrazolium red or tetrazolium violet, 50 μg ml^{-1}). Best results were obtained when concentrated stocks of M9 (without magnesium), magnesium sulphate, amino acids, carbon source, and other additives were combined on a Petri plate with freshly melted water agar. Reagents other than agar were purchased from Sigma. The strains, carbon sources, and vital dyes used for this figure were: *a*, HCB317 (*tsr*, ref. 21), succinate (2.2 mM), tetrazolium red; *b*, HCB317, fumarate (3 mM), tetrazolium violet; *c*, RP437 (wild-type, ref. 22), fumarate (2 mM), tetrazolium violet; *d*, RP4368 (*tsr*, from J. S. Parkinson), succinate (2.2 mM), tetrazolium violet. Plates were incubated at 25° C for 72 h. They were photographed with a Tektronix C-30A oscilloscope camera on Polaroid type 667 film against a flat-back background, with illumination provided slantwise from below by a stack of two 22 W 8-inch Circline fluorescent lamps mounted 180° out of register.

at the strip chart, as expected. Within about 5 min after the second addition (at c), however, cells moved back from chamber 2 into chamber 1, as indicated by the negative slope. This occurred even though the density of cells in chamber 2 was only about 2% of that in chamber 1. From the peak concentration of cells in chamber 2 and the slope of the curve between 43 and 73 min, the chemotaxis drift velocity was estimated to be 4.8 $\mu m\ s^{-1}$. This experiment was repeated in the presence of 50 mM α-methyl-D,L-aspartate (enough to saturate the aspartate receptor), as shown in the inset, Fig. 4. Now, following the addition of hydrogen peroxide at (b) and (c), the cells continued to move from chamber 1 into chamber 2, if anything, at a slightly higher rate. Two other control experiments were done with wild-type cells (strain AW405) grown on tryptone and suspended in motility medium[14]. Cells moved back from chamber 2 into chamber 1 at a rate similar to that shown in Fig. 4 on addition of about 2 μM L-aspartate to chamber 1. When, instead, hydrogen peroxide was added to both chambers, the results were identical to those shown in the inset of Fig. 4. From these experiments we conclude: (1) that a potent attractant was excreted by cells in α-ketoglutarate following exposure to hydrogen peroxide (equivalent to about 2 μM L-aspartate); (2) that this attractant was of low molecular weight (with a diffusion coefficient of order $10^{-5}\ cm^2\ s^{-1}$, as judged by the short time interval between the addition of peroxide and the change in sign of the cell flux); and (3) that the response to this attractant could be blocked by saturation of the aspartate receptor.

If the attractant is L-aspartate and 10^7 cells dump their cytoplasmic allotment into 1 ml of the external medium, what would the intracellular concentration have to be to give a final concentration of 2 μM? Assuming a cytoplasmic volume of order $10^{-12}\ cm^3$ per cell, the answer is 0.2 M. So either the cells are heavily loaded with aspartate or the attractant is more potent than aspartate. It cannot be much more potent, because *E. coli* only responds to attractants when it can measure changes in concentration that occur over a span of few seconds. To make this measurement with adequate precision, a cell has to count a large number of molecules. The fractional error in this count

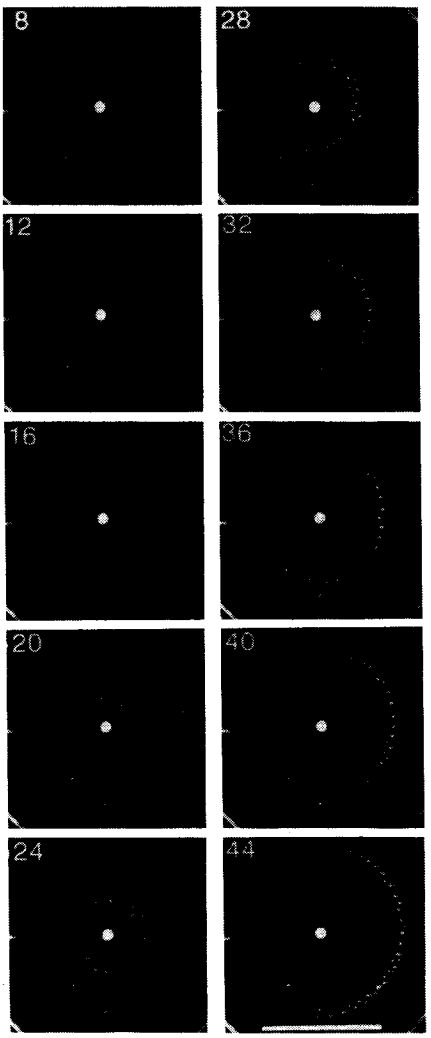

FIG. 2 Generation of a sunflower-like array. Images from a time-lapse video recording are shown at intervals of 4 h, beginning 8 h after inoculation. The labels indicate the elapsed time. Bar: 4 cm.
METHODS. Cells of *E. coli* strain HCB317 were incubated as in Fig. 1, except on a larger volume of medium (10.8 ml in an 8.5-cm Petri plate) containing 0.29% agar, 4.5 mM succinate, and no vital dye. The images were recorded with a Dage-MTI Series 68 Newvicon camera on a JVC Model BR-9000U video cassette recorder. The illumination was from below and the right.

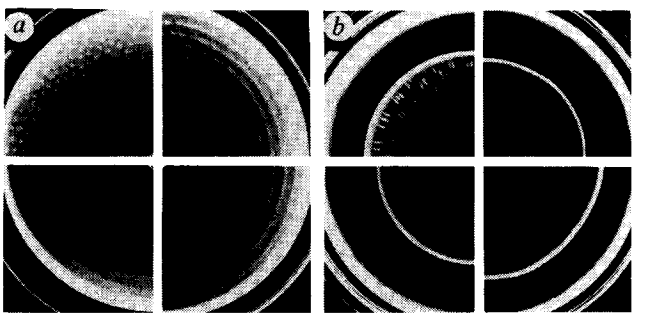

FIG. 3 Inhibition of pattern formation by the non-metabolizable aspartate analogue α-methyl-D,L-aspartate. Two series of experiments are shown, with the control in the upper left-hand quadrant and the concentration of inhibitor increasing clockwise. *a*, A pattern similar to that of Fig. 2; *b*, a pattern combining elements of Fig 1*b* and *d*. In *a* the concentration of α-methyl-D,L-aspartate was increased from 0 to 16, 31 and 125 μM, respectively; at 250 μM (not shown), the pattern disappeared (conditions as in Fig. 2, 72 h). *b*, The concentration was increased from 0 to 25, 75, and 125 μM, respectively. At 250 μM (not shown), the ring at the periphery was more diffuse, and the spreading rate decreased; at 500 μM, the ring disappeared (conditions as in Fig. 1*b* but with 2.5 mM succinate, 36 h).

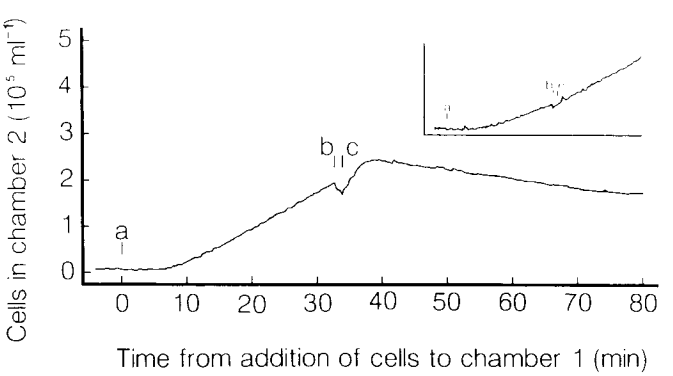

FIG. 4 The number of cells per unit volume in chamber 2 of the chemotaxis apparatus, recorded as a function of time. At (a) cells were added to chamber 1. At (b) and (c) equal amounts of hydrogen peroxide were added to chambers 2 and 1, respectively. Inset: a control experiment run in the presence of 50 mM α-methyl-D,L-aspartate, shown half scale.
METHODS. Cells of *tsr* strain HCB317 were grown at 30°C without aeration on the medium described in Fig. 1 (without agar or vital dye) on 5 mM α-ketoglutarate to a density of 9×10^7 cells ml^{-1}. A filtrate of part of this culture was used to fill the two chambers. The addition at (a) was 0.20 ml unfiltered culture, chased with 0.15 ml filtrate, yielding a final density of 10^7 cells ml^{-1}. The additions at (b) and (c) were 18 μl of 0.9 M hydrogen peroxide, chased with 0.15 ml filtrate, yielding final concentrations of 9 mM. The apparatus was taken apart, cleaned, and reassembled, and the experiment was repeated with a different culture to which 50 mM α-methyl-D,L-aspartate had been added (inset). The microchannel plate used in these experiments had 10 μm pores and was 1 mm thick. The experiments were done at 25° C.

is inversely proportional to the square root of the product of the concentration and the integration time[15]. One can show that attractants can work well in the nanomolar but not in the picomolar range.

The formation of complex patterns by chemotactic cells of *E. coli* provides a striking example of biological self-organization by interacting, initially identical, microscopic elements. This occurs in other unicellular organisms in response to environmental stress. Examples include formation of sporangiophores by the social bacterium *Myxococcus xanthus* or slugs by the cellular slime mould *Dictyostelium discoideum*, both triggered by starvation at high cell densities[16]. With *E. coli*, aggregation is not a specific stage in a developmental cycle; however, it could rapidly alleviate stress due to generation of hazardous byproducts in respiration, simply by reducing the local oxygen concentration. Unlike the well-known travelling waves of aggregating cells of *D. discoideum*[16] or the ripples of *M. xanthus*[17], the structures formed by *E. coli* are temporally stable. This is true for periods of at least half an hour in liquid, where the cells remain motile. It is true for days in agar (Figs 1 and 2), because the cells become nonmotile. The reasons for this loss of motility are not known.

With *E. coli*, as well as with *D. discoideum*, the cells migrate in gradients of autoregulators that they excrete themselves. Pattern formation by this mechanism is thought to occur in more complex developmental systems, although its importance is subject to debate[18]. With *E. coli*, it should be easier to define the relevant parameters. An apt mathematical analysis appears to be that of Oster and Murray[19]. In their model, cells move towards the source of a chemical attractant that has a finite lifetime, excreted at a rate that saturates with cell density. Bifurcation analysis dictates the formation of spots or stripes. However, we do not know how the excretion of our attractant depends on cell density or time, or whether this substance is stable. The most interesting unanswered questions concern the identity of the chemical attractant and its mode of production. □

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Demonstration by NMR of folding domains in lysozyme

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ALTHOUGH there has been much speculation on the pathways of protein folding, only recently have experimental data on the topic been available. The study of proteins under conditions where species intermediate between the fully folded and unfolded states are stable has provided important information, for example about the disulphide intermediates in BPTI[1,2], *cis/trans* proline isomers of RNase A[3] and the molten globule state of α-lactalbumin[4]. An alternative approach to investigating folding pathways has involved detection and characterization of transient conformers in refolding studies using stopped-flow methods coupled with NMR measurements of hydrogen exchange[5,6]. The formation of intermediate structures has been detected in the early stages of folding of cytochrome *c* (ref. 7), RNaseA[8] and barnase[9]. For α-lactalbumin, hydrogen exchange kinetics monitored by NMR proved to be crucial for identifying native-like structural features in the stable molten globule state[10]. An analogous partially folded protein stable under equilibrium conditions has not been observed for the structurally homologous protein hen egg-white lysozyme, although there is evidence that a similar but transient state is formed during refolding[4,11]. Here we describe NMR experiments based on competition between hydrogen exchange and the refolding process which not only support the existence of such a transient species for lysozyme, but enable its structural characteristics to be defined. The results indicate that the two structural domains of lysozyme[12,13] are distinct folding domains, in that they differ significantly in the extent to which compact, probably native-like, structure is present in the early stages of folding.

The refolding experiment involved dilution of droplets of protein denatured in guanidinium chloride (GuHCl) in H_2O solution into a denaturant-free solution of D_2O. This initiated refolding and hydrogen exchange simultaneously. After folding was complete, exchange was quenched by lowering the pH, and the two-dimensional J-correlated (COSY) NMR spectrum of the refolded protein was compared with a control in which the protein was not denatured. The competition between folding and exchange can be adjusted, for example by varying the pH[14]; for our study, pH values in the physiological range (6.5-8.5) were found to be appropriate.

The refolding populations of lysozyme consist of rapid and slow folding species[15-17]. The latter, presumed to be the result of *cis/trans* proline isomerism, is present as only 10% of the unfolded population, and was not expected to make significant contributions in our experiment. Refolding of the former results in some secondary structure formation in milliseconds[4,18] with complete refolding occurring in seconds[11,16]. The hydrogen-deuterium exchange observed in the competition method represents an integral of the exchange over the time of protein folding. As an approximation, a single kinetic step model can be applied to either the early phase or the late phase of the folding (Fig. 2*b*). In the first case, we assume no exchange after the initial collapse of the protein. In the second case, we assume that the rate of initial collapse is far greater than the exchange rate in the unfolded state. The degree of exchange, for an individual amide, depends on the rate at which the protein protects the amide from exchange, relative to the rate of exchange of the unfolded (first case) or collapsed (second case) protein states. These two cases cannot be distinguished because direct measurements of rates are not made in this experiment.

The competition method has several characteristics that are useful for the present study. First, it does not rely on the thorough