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Biophysical Chemistry 101–102 (2002) 133–144

Biophysical Chemistry

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Quantitative urea gradient gel electrophoresis for studies of dissociation and unfolding of oligomeric proteins☆

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Received 7 February 2002; accepted 21 March 2002

Abstract

Urea gradient gel electrophoresis combined with quantitative image processing of stained gels was used to analyze the dissociation and unfolding of the catalytic subunit of aspartate transcarbamoylase. The subunit, composed of three identical polypeptide chains, dissociates reversibly at high urea concentrations into unfolded chains. A comparison of the complex, but reproducible, gel patterns obtained for the native subunit and for the denatured protein in 6 M urea revealed significant differences at intermediate urea concentrations due to the presence of a transient kinetic intermediate identified as a relatively compact monomer. Mass transport equations based on a three state model were used to describe the urea gradient gel electrophoresis experiments, and a numerical solution yielded estimates of the population of molecular species and kinetic constants for the unfolding and refolding reactions as well as the dissociation and reconstitution reactions.

Keywords: Urea gradient gel electrophoresis; Denaturation and reconsitution of oligomeric proteins; Unfolding and folding of polypeptide chains; Aspartate transcarbamoylase and catalytic trimers; Analysis of patterns by mass transfer equations

Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit or trimer; N_3, alternative description of C subunit; *M*, folded monomer; *U*, unfolded polypeptide chain; *R*, regulatory subunit or dimer; TEMED, N, N, N′, N′-tetramethylethylenediamine; TCA, trichloroacetic acid; c, urea concentration; R_f, electrophoretic mobility

☆ In dedicating this article to honor Professor John Schellman, we would like to express our admiration and thanks for his remarkable contributions to present knowledge of the physical chemistry of proteins.

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0301-4622/02/$ - see front matter
PII: S0301-4622(02)00154-0

1. Introduction

Techniques such as fluorescence and circular dichroism, have been invaluable in studies of the denaturation of proteins, but it is recognized that these methods yield parameters representing some average of the individual contributions from the various species in the samples. Since the denaturation of globular proteins is a complex process involving the existence of slow- and fast-folding molecules with transient intermediates along the folding pathway [1–6], a technique is required which provides quantitative information about the amounts and identities of the different components in the population of molecules. Among the methods capable of selectively detecting chemically identical but conformationally different molecules, urea gradient gel electrophoresis ranks as one of the most convenient and inexpensive procedures. As shown in the work reported here, this method has great potential for the determination of the mechanism and the rates of the individual steps in the reversible denaturation of oligomeric proteins.

Upon denaturation of oligomeric proteins at least two different processes, dissociation and unfolding, occur leading to the possible coexistence of at least three different molecular species, native protein, dissociated monomers and unfolded polypeptide chains. There also may be partially unfolded but still intact oligomers. Dissociation and unfolding in general are structurally coupled and the processes overlap kinetically. As a result, the transformations, oligomer → monomer and monomer → unfolded chain, often appear experimentally as a two-state transition, oligomer → unfolded chains. The denaturation of dimers of Arc repressor [7], for example, can be described by a two-state transition model $N_2 \rightleftarrows 2U$, where N_2 represents the native dimer and U corresponds to unfolded polypeptide chains. For such a transition it is implied that quaternary interactions are important for stabilization of the folded monomers [8]. However, partially structured monomers as well as denatured dimers occur transiently and can be observed using appropriate methods [9–11].

The application of urea gradient gel electrophoresis for studies of protein unfolding and refolding is based on the observation that the relative mobility (R_f) of a protein in polyacrylamide gels depends on both the hydrodynamic size and net electrostatic charge of the polypeptide chain. In urea gradient gel electrophoresis, the effective size of a protein molecule changes significantly along the transverse linear gradient of urea concentration due to the urea promoted unfolding, whereas the net charge, as a first approximation, is not directly affected by urea. Thus, unfolded protein migrates more slowly than native protein thereby allowing monitoring of urea promoted conformational transitions by measuring R_f as a function of urea concentration. Through the separation of structurally different molecules in the gel, urea gradient electrophoresis is potentially capable of detecting and identifying quantitatively transient kinetic intermediates populated during unfolding and refolding of the protein, which are undetectable in equilibrium experiments.

Previous studies [12] demonstrated that urea gradient electrophoresis was a robust technique for visualizing various molecular fractions co-existing in solution during the conformational transition of a protein. In addition, it was potentially useful for estimating kinetic constants for the interconversion between different conformations when the rates of the various processes were sufficiently slow [13]. It has been stressed that accuracy in the analysis of the patterns along with the consistency and reproducibility of the urea gradients in the gels are critical for the quantitative determination of the protein distribution in urea gradient gel electrophoresis.

The compact, enzymatically active C subunit of *Escherichia coli* aspartate transcarbamoylase (ATCase, carbamoylphosphate: L-aspartate carbamoyl transferase, E.C. 2.1.3.2), consists of three identical polypeptide chains with molecular weights of 34 000. It is readily and reversibly dissociated into both folded and unfolded monomers [14–16]. In the presence of urea, C trimers (N_3) dissociate to compact monomers (M) and to unfolded polypeptide chains (U) between 2 and 5 M urea with the midpoint near 3.5 M

Urea gradient gel electrophoresis patterns obtained with both native C trimers and fully denatured protein have been analyzed to detect the fraction of folded (or partially folded) monomers,

M, and to characterize the dissociation—unfolding process as well as the refolding—reconstitution reactions. The kinetics of the dissociation of trimers to monomers and urea-promoted conformational changes in the molecular species have been derived from the rates of migration through the gel. In addition, a comparison of the patterns for the native and denatured proteins provided valuable information in terms of the kinetics of denaturation and reconstitution.

Computer simulation of urea gradient electrophoresis experiments based on numerical solution of non-linear differential equations describing transport of interacting molecules [17,18] demonstrated the validity of the three-state model for denaturation of C subunit of ATCase. The fitting of the experimental urea gel profiles to the computed model produced estimation of the equilibrium and kinetic parameters for both the urea promoted dissociation of trimers into folded monomers and the unfolding of monomers.

2. Materials and methods

2.1. Purification of protein

The two-step procedure employed for the purification of ATCase is based on the remarkable selectivity of the Ni^{2+}–NTA agarose (Qiagen Inc.) for proteins with an affinity (his_6) tag [19]. ATCase carrying six additional histidine residues on the N-terminus of the regulatory chains [20] was overproduced in *E. coli* grown in LB medium containing 50 mg/l ampicillin. The collected cells were resuspended in 50 mM Tris–Cl, 200 mM KCl, pH 8.8, followed by sonication, and the clarified cell extract was loaded on a Q-Sepharose column equilibrated with the same buffer. ATCase was eluted with 0.66 M KCl, 50 mM Tris–Cl, pH 8.8 and loaded on a Ni–NTA column equilibrated with the same buffer. The fractions of ATCase eluted with 0.3 M imidazole were 95% pure as detected by native and SDS polyacrylamide gel electrophoresis. Filtration on a SuperDex column (Pharmacia) was used as a final step in order to obtain ATCase of higher purity.

Purification of C subunit was performed by treatment of ATCase with neohydrin [21] followed by separation of C and R subunits on Ni^{2+}–NTA column. The unbound fraction contained 99% pure C trimer. It was transferred to the 20 mM Tris–Cl buffer at pH 7.5 using Sephadex G-25 as a small desalting column (Pharmacia) and used for unfolding/refolding experiments.

2.2. Preparation of urea gradient gel

Transverse urea gradient gels were prepared according to the procedure described earlier [12,13] with minor modifications. The 10×8 cm gel plates with 1.5 mm spacers were placed in the casting box in the orientation turned 90° relative to the direction of electrophoresis. Two 5% acrylamide gel solutions (acrylamide–bisacrylamide ratio 19:1) in 0.375 M Tris–Cl buffer, pH 8.8 containing 0 M and 6 M urea were mixed using a linear gradient former. In order to use the natural density of the urea and acrylamide solutions to stabilize the gradient, the overlay buffer (0.375 M Tris–Cl, pH 8.8) was first fed into the bottom of the casting box. This was followed by the acrylamide solution with no urea, then by the linear gradient of urea and finally by more of the solution with 6 M urea [22]. The amounts of ammonium persulphate and TEMED were adjusted to bring the polymerization time to approximately 1 h. Polymerized gels were stored at 4 °C and used within 24 h.

2.3. Urea gradient electrophoresis

Protein samples containing 6 M urea for gradient electrophoresis of unfolded protein were prepared by mixing of the protein solution (2–3 mg/ml) in 20 mM Tris–Cl buffer, pH 7.5 with the 8 M urea solution in the same buffer. The sample was incubated at 0 °C for at least 4 h before loading so as to allow denaturation of the protein to form unfolded polypeptide chains.

Electrophoresis was performed in the cold room at a constant current of 10 mA per gel. The 25 mM Tris and 150 mM glycine, pH 8.5 running buffer in combination with 375 mM Tris–Cl, pH 8.8 gel buffer was found to give high quality electrophoresis patterns. Typical times of electrophoresis were approximately 2.5 h, but longer runs

up to 12 h were also performed. Protein in the gel was visualized by staining with 0.01% Coomassie blue G-250, 12.5% TCA.

2.4. Gel image processing

Stained gels were digitized in 16 bit gray-scale mode using Epson EC-1000C image scanner. Gel images were stored in the computer as 250×350 2D-arrays of amplitude, A, corresponding to values proportional to optical density. Further processing of the image was performed with 'NIH-Image' software (available at the Web site http://rsb.info.nih.gov/nih-image/) which included background subtraction, contrast enhancement, dye front baseline correction, and signal-to-noise enhancement. The 1D-profiles of A vs. relative electrophoretic mobility, R_f, sampled with 0.5 M urea increment, were analyzed with ORIGIN software (MicroCal Inc.) using standard algorithms for curve fitting and analysis, such as smoothing, filtering, deconvolution, and integration.

In order to use quantitative values of the band intensity as a measure of the amount of protein, the intensity scale had to be calibrated. This calibration was performed using standard native gel electrophoresis of C subunit under conditions close to those used for urea gradient electrophoresis. The intensity of the bands, as measured by the area under the peak, plotted vs. the amount of protein exhibited linear behavior up to 10 μg per 0.5 cm lane, which corresponds to approximately 150 μg per gel–wide load. Intensity scans of the individual bands were close to Gaussian in shape with slight asymmetry (backward tailing), which were more pronounced for heavily loaded lanes.

3. Results and discussion

3.1. Analysis of experiments on native and denatured C trimers

Model calculations of urea gradient gel patterns for monomeric proteins [18] have revealed that a transition with a time constant comparable to the duration of electrophoresis produces a 'smear' between the bands of native and unfolded protein. If the rates of interconversion are very slow compared to the rate of separation of the species, the individual components will be observed. Conversely, for those systems in which interconversion is very rapid, a single band is observed with a mobility representing the average of the different species. Since in the studies described here, there were dissociation as well as unfolding reactions and their reverse, it was important to conduct urea gradient gel electrophoresis experiments on both the native and denatured proteins. The transition midpoint (urea concentration, $c_{m,}$ at which $[M] = [U]$) obtained in the refolding and unfolding experiments might be different from those observed under equilibrium conditions because of systematic underestimation of the unfolded fraction in unfolding experiments and the refolded fraction in refolding experiments.

Two simultaneous complementary urea gradient gel electrophoresis experiments were performed for all studies. In the unfolding experiment, native C subunit, which was applied to the gel in 20 mM Tris–Cl buffer, undergoes denaturation during the electrophoretic migration. For the refolding experiments, protein was applied to the gel in the denaturing buffer containing 6 M urea. During electrophoresis, refolding of the chains and association occur as the protein migrates into regions of low urea concentrations, where the native structure predominates. The combined denaturation, $N_3 \rightarrow 3U$, and reconstitution, $3U \rightarrow N_{3,}$ transitions of C trimers are known to have relatively slow kinetics, which accounts for the discontinuous character of the electrophoresis patterns. Moreover, the rates of the various processes vary as a function of the urea concentration. Hence, it is to be expected that there will be variable kinetics throughout the urea gradient, and that different patterns will result depending on whether native or denatured protein is applied.

Representative patterns obtained with native and denatured C subunit are shown in Fig. 1a,c. The protein band migrating with R_f approximately 0.9 corresponds to native C trimers (N_3 band). A second band with R_f approximately 0.2 in 6 M urea and increasing to 0.6 in 0 M urea corresponds to monomers (M band) formed by the dissociation process. The assignment of bands was confirmed by staining the urea gradient gels for enzymatic

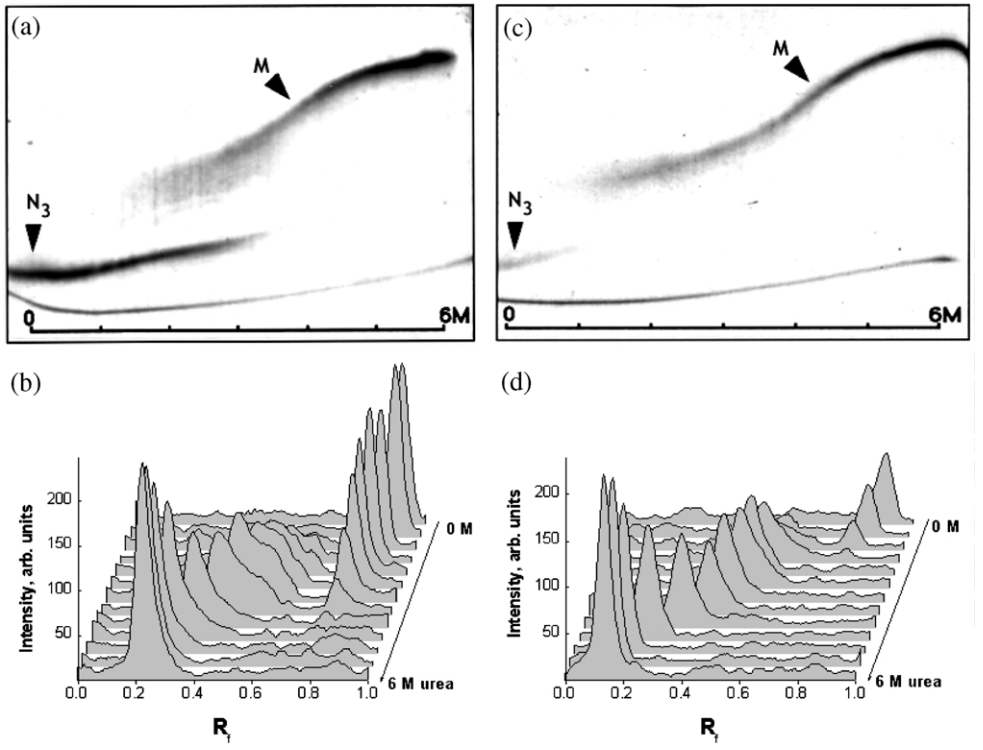

Fig. 1. Urea gradient gel electrophoresis patterns of C subunit of ATCase. (a) Image from experiment with native protein applied at the top of the gel and a downward migration direction. The component with highest migration rate, observed at the lower urea concentrations, corresponds to native C trimers (N_3), and the other component with changing mobility corresponds to monomers (M) which are converted from the compact form to unfolded chains at intermediate urea concentrations. (b) Digitized and processed image of stained gel. The intensity of the various bands is shown in arbitrary units as function of the urea concentration and the R_f of the components. (c) Gel image obtained when denatured protein, incubated in 6 M urea for 4 h at 4 °C, protein was applied to the gel. (d) Digitized and processed image. Gel images were scanned in 16 bit gray scale and sampled as intensity profiles in 0.5 M increments from 0 to 6 M urea.

activity (data not shown) [23]. Only the N_3 band exhibited activity in agreement with earlier findings that dissociation of C trimers leads to complete inactivation [14,15].

The main features of the electrophoresis patterns, in terms of the positions of the bands and their relative intensities, were checked for reproducibility by repeated experiments leading to the conclusion that the precision of data was within ± 0.2 M of urea concentration and ± 0.03 of R_f units. Processing of the stained gels for intensities and positions of the bands led to the digitized images shown in Fig. 1b for electrophoresis of the native C trimer and in Fig. 1d for the denatured protein. The images in Fig. 1a show clearly a preponderance of C trimer at the start of the experiment and followed by its disappearance as the protein migrates into the higher urea concentrations. There is a concomitant formation of folded monomers followed by the appearance of unfolded polypeptide chains. In contrast, the experiment in which denatured protein was applied to the gel (Fig. 1c) shows at the top of the urea gradient a considerable amounts of protein with low mobilities as well as a principal component having an R_f corresponding to intact C trimers. In the course of the migration of the protein into higher urea concentrations, the patterns for the denatured protein begin to resemble those for the native C trimer with the unfolded polypeptide

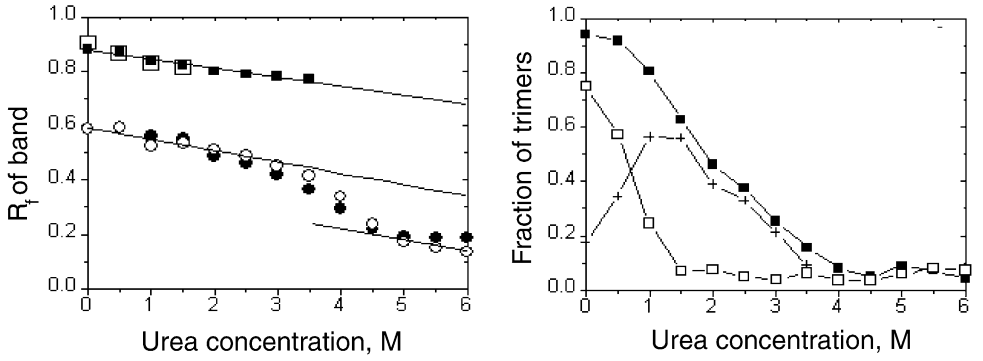

Fig. 2. (a) Plot of R_f at the band maximum vs. urea concentration. Squares represent C trimers observed when native (■) or denatured (□) protein was applied to the gel. Circles correspond to the band of monomers, compact or unfolded, observed when native protein (●) or denatured protein (○) was applied to the gel. (b) Fraction of trimers, estimated as normalized intensity of the N_3 band, plotted vs. urea concentration. Solid symbols (■) refer to the population of trimers when the native protein was loaded to the gel and open symbols (□) refer to the experiment with denatured protein. The crosses (+) represent the differences in the fraction of trimers observed in the two experiments.

chains becoming the predominant product. These analyses of the urea gradient gel electrophoresis experiments were in reasonable agreement with conclusions inferred from circular dichroism or fluorescence measurements and enzymatic activity assays (unpublished data).

3.2. Characterization of trimers and monomers in urea gels

Fig. 2a shows the dependence of the R_f values on the concentration of urea. Whereas the R_f for the trimer varies only slightly as a function of urea concentration, there is a marked change in the mobility of the monomer at urea concentrations between 3 and 5 M. The small, linear decrease in R_f for the intact trimer at varying urea concentrations indicates the absence of any significant change in compactness or major conformational transition in that protein, and it is consistent with observations on many proteins studied by this method [12,13]. This slight change in mobility is presumably caused by the varying dielectric constant of the gel due to the gradient of urea concentration.

Unlike intact C subunit, the monomers undergo a cooperative unfolding transition, shown in Fig. 2a, that appears as a broad sigmoidal change in R_f with a midpoint approximately 4 M urea. The mobilities of fractions estimated in unfolding and refolding experiments were superimposed in one plot to demonstrate the consistency of the data. Minor difference observed mostly in the transition region seems to be caused by ambiguity in assigning the position of the maximum due to the broad and complex shape of the M band in this range of urea concentration. It is of interest that the mobility of the *M* band, at urea concentrations approximately 1–2 M, is less than that of the trimers. Thus, there appears to be a loss of compactness upon dissociation of the trimers prior to the unfolding of the polypeptide chains at the higher urea concentrations.

3.3. Distribution of protein as trimers and monomers

Information relevant to evaluating the kinetics and equilibria involving the various species can be deduced from the gels by measurements of the amounts of the different components. These amounts were determined by integration of the patterns such as those shown in Fig. 1b,d. The resulting areas evaluated from the intensity of the bands thus provided a direct measurement of the amounts of the various components at the different urea concentrations. For those experiments in which the bands of different components overlap,

a deconvolution procedure was applied to give individual Gaussian peaks for estimating the amount of each component contributing to the intensity of the entire band. In principle, the amount of protein determined in this way should be constant throughout the gel. In practice, however, the total amount summed for the different components was found to vary significantly through the gel. These variations could be attributed to different experimental limitations such as uneven loading of sample on top of the gel and non-uniform staining of the gels. In addition some protein aggregation occurs mostly at intermediate urea concentrations, and there are constraints in the algorithm for background subtraction that arise from ambiguities in discriminating between signal and background.

Despite the limitations in obtaining quantitative data for the distribution of the various species, it was possible to determine the fraction of protein in the form of trimers at the different urea concentrations for both the experiments starting with intact trimers and with the denatured protein. The reversible cooperative transition, $N_3 \rightleftarrows 3M$, is illustrated by the results in Fig. 2b. It is clear that different results are obtained for the denaturation experiment and that involving refolding and association reactions. Some of that difference is almost certainly attributable to the inability of all the unfolded polypeptide chains to refold into the proper conformation for association into trimers. As a result of misfolding of some of the chains, aggregates form thereby leading to a decrease in the yield of trimers in the folding experiment. There are additional complications resulting from the slow rates of the folding and association reactions. If the electrophoretic migration was much slower, one would expect these two transitions to merge in a single graph corresponding to the equilibrium dissociation of C trimers. However, the quality of the gel patterns gradually deteriorates with the increase in duration of the experiment presumably due to protein aggregation.

3.4. Kinetic effects

As indicated above, the differences in the population of trimers, estimated from the experiments with native and denatured protein (Fig. 2b), is attributable in part to the slow kinetic processes. It would be expected, therefore, that larger differences would be expected under conditions leading to slower kinetics. The maximum difference in the fraction of trimers observed in the electrophoresis experiments (Fig. 2b) occurs at 1.3–1.4 M urea which provides a rough estimate of the midpoint of the equilibrium unfolding transition where unfolding and folding reactions are likely to be very slow. There also may be variations in the gel patterns depending on the amount of protein loaded onto the gel because the rate of association of monomers into trimers depends on the protein concentration.

Since the transition curves for $M \rightleftarrows U$ in Fig. 2a are continuous and virtually identical when obtained in the unfolding and refolding experiments, we can conclude that the folding–unfolding process involves relatively fast kinetics. Thus, it is the $N_3 \rightleftarrows M$ reaction that is the rate-limiting step in both denaturation and reconstitution of C trimers.

The gel profiles in Fig. 1 show that the bands are more symmetric closer to the edges of the gel, where the kinetics are fast. More asymmetric bands are observed in the patterns representing the slower interconversion between fractions. This asymmetry was observed primarily for the *M* band, indicating further the significant differences between the overall kinetic rates of dissociation and reassembly of trimers.

In the course of their migration through the gel, dissociating trimers are continuously enriching the fraction of monomers, causing asymmetry of the band, *M*, corresponding to the monomers. The N_3 band remains symmetric at any specific urea concentration because the yield of reassembled trimers is negligible due to the extremely slow kinetics, and the shape of the band is readily fit to the Gaussian function $G(x)$. We assume that at any time, *t*, the asymmetric band representing *M* can be approximated by exponential decay of the migrating (N_3) band represented by the Gaussian as shown in Eq. (1),

$$A_c(x) = \int G(x-t) \bullet \exp(-k_{dis}t)dt \quad (1)$$

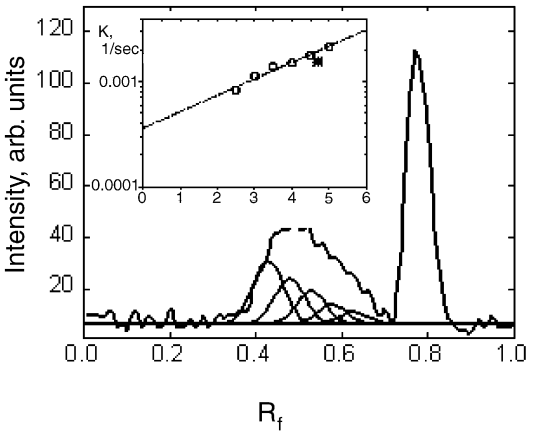

Fig. 3. Approximate fitting of the asymmetric profile of the *M* band as function of R_f in the region of 2.5 M urea in an experiment with native C trimers. The intensity of the complex, asymmetric profile was fit by deconvolution into a series of Gaussian curves based on the half-width of the symmetric N_3 band and the exponential decay of the trimers as described in Eq. (1) (dash-line peaks) with the kinetic exponent for dissociation of the triners. The insert shows the estimated kinetic constants for dissociation of C trimers at various urea concentrations. The star represents the value obtained from circular dichroism measurements.

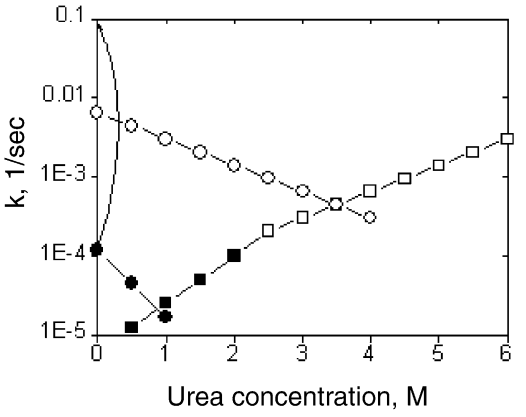

Fig. 4. Kinetic constants evaluated from the simulations of the urea-gradient gel experiments as a function of urea concentration. Dissociation constant, k_{dis} (■); reconstitution constant, k_{rec} (●); unfolding constant, k_{unf} (□); refolding constant, k_{ref} (○). All the constants, except k_{rec}, were assumed to be linear functions of urea concentration, since they represent monomolecular reactions. The k_{rec} at 0 M urea is larger than the value estimated by linear extrapolation.

where x is a migration coordinate, and k_{dis} is the rate constant for dissociation. Eq. (1) is a convolution of the Gaussian, $G(x)$, representing the distribution of non-converting molecules in the gel profile along with a correction allowing for the migration and for the kinetic interconversions occurring in the system. The latter is represented by the exponential term containing the rate constant for the dissociation of trimers. This formulation results in an asymmetric profile, $A_c(x)$, and a reasonably good approximation for the shape of the *M* band. At high urea concentrations where k_{dis} is large, it becomes $G(x)$ for the fast reaction. Fitting the profile for the monomer band to Eq. (1) gives an estimate of k_{dis} as a function of urea concentration (Fig. 3). As expected for this process, $\log(k_{dis})$ shows a linear dependence on urea concentration. Estimated values are in good agreement with circular dichroism data for the kinetics of unfolding of C subunit (unpublished results).

3.5. Simulation of urea gradient electrophoretic patterns

Transport of dissociating macromolecules, undergoing reversible unfolding during electrophoresis presents no difficulties in interpretation if the rates of reactions are either sufficiently fast, or slow, relative to the time of the separation of species in the experiment [17]. For processes involving very rapid reactions, the transport patterns at any time would correspond to the equilibrium mixture of species at each concentration of urea. In contrast, systems involving very slow reactions would give a pattern corresponding to non-interacting macromolecules in amounts represented by the starting conditions and with electrophoretic mobilities of the individual species. The situation is considerably more complex, however, when re-equilibration between species occurs during their differential transport in the time scale comparable to the rate of electrophoretic separation of the species. In this case, peaks in the transport pattern cannot be placed unambiguously into correspondence with individual reactants or products, and the transport equations must be solved in the most general form.

Electrophoresis of the system containing C trimers, N_3, relatively compact monomers, M, and unfolded chains, U, can be described by the following model,

$$N_3 \underset{k_{rec}}{\overset{k_{dis}}{\rightleftarrows}} 3M \underset{k_{ref}}{\overset{k_{unf}}{\rightleftarrows}} 3U \tag{2}$$

The changes in the concentrations of the different species with time, t, and position, x, in the gel at each urea concentration from 0 to 6 M are described by the set of transport equations which account for the migration rates of the species as well as their interconversion:

$$\frac{\partial[N_3]}{\partial t} = -V_N \frac{\partial[N_3]}{\partial x} - k_{dis}[N_3] + k_{rec}[M]^3; \tag{3}$$

$$\frac{\partial[M]}{\partial t} = -V_M \frac{\partial[M]}{\partial x} - k_{rec}[M] - k_{unf}[M] + k_{dis}[N_3] + k_{ref}[U]; \tag{4}$$

$$\frac{\partial[U]}{\partial t} = -V_U \frac{\partial[U]}{\partial x} - k_{ref}[U] + k_{unf}[M]; \tag{5}$$

where k_{dis}, k_{rec}, k_{unf} and k_{ref} are kinetic constants of dissociation, reconstitution, unfolding and refolding respectively; V_N, V_M and V_U are migration velocities for the trimers, compact monomers and unfolded polypeptide chains, respectively. In order to reduce the computational complexity, we used programming algorithms with linear concentrations of all the species. This is certainly an oversimplification for the reconstitution reaction, but apparently this approximation affects only the reconstitution kinetic constant, k_{rec}, in Eq. (3) without any effect on the other parameters. It is also assumed that diffusion is slow and can be taken into account by appropriate setting of starting conditions. At time, $t=0$, the protein distribution in the gel is represented by the Gaussian with a half-width derived from experimental profiles. The validity of this assumption is justified in part by the observation of equal half-widths of the bands of catalytic trimers at 0 M urea and unfolded monomers at 6 M urea. Two simulations with different starting conditions were performed for each experiment. In the first, for which 0.3 mg/ml of native protein was applied to the gel, $N_3=0.3$, $M=0$, and $U=0$ at $t=0$. For the second simulation in which 0.3 mg/ml of denatured protein was applied to the gel, $N_3=0$; $M=0$; and $U=0.3$ at $t=0$. Solving the equations involved the obvious constraints that all concentrations were greater than zero and that the total concentration of protein was accounted for by the sum of $[N_3]+[M]+[U]$.

Simulations of two-dimensional urea gradient gels were performed on a Silicon Graphics Indigo II workstation. The program package URGEL, written for standard UNIX environment, implements numerically stable algorithms for solving a system of non-linear differential equations of third order. The program solves the system of Eq. (3) Eq. (4) Eq. (5) at various urea concentrations evenly spaced from 0 to 6 M in increments of 0.5 M. The solutions $[N_3(x,t), M(x,t), U(x,t)]$ were found to be stable when the sampling interval Δx was smaller than 0.3 mm (260 points per 80 mm gel) and $\Delta t<0.1$ min (2000 points per 2.5 h run) with the condition $V\Delta t/\Delta x<0.2$ for any V. The velocities, V, were assumed to be linear functions of the urea concentration, c, estimated from plots of R_f vs. urea concentration in Fig. 2.

The four kinetic constants varying exponentially with the urea concentration were considered as the parameters affecting the protein distribution pattern in the gel most conspicuously. For two state transitions, the logarithm of the kinetic constant can be considered as a linear function of the free energy change, and the logarithm of the constant was assumed to change linearly with urea concentration, as shown in Fig. 4. The starting values for k_{unf} and k_{ref} were those obtained from circular dichroism measurements of the kinetics (unpublished results). The other two, k_{dis} and k_{rec}, were roughly estimated from kinetics of activity loss and restoration in the presence of urea. Finally, all four constants were adjusted to give the best fit to the experimental gel patterns in Fig. 1 for both native and denatured protein applied to the gel.

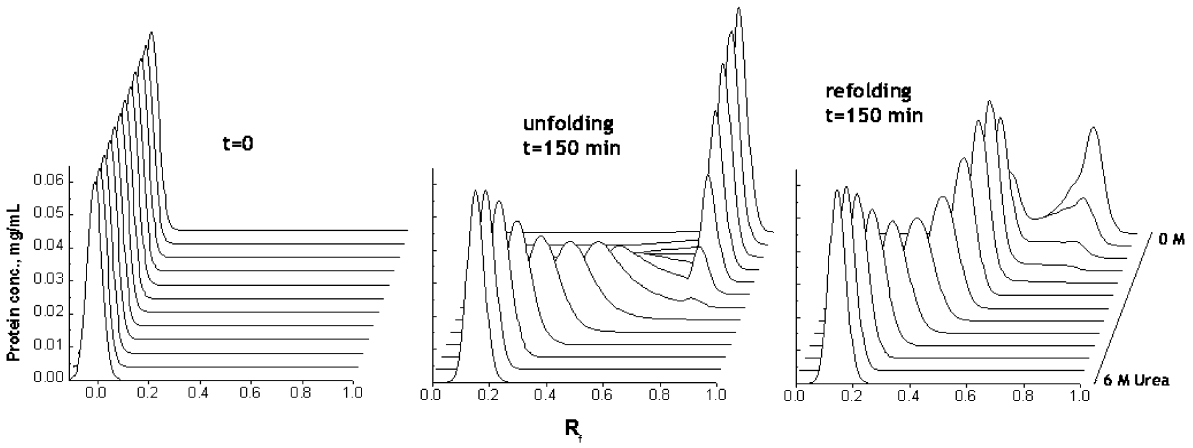

Fig. 5. Computer generated profiles of the protein distribution in urea gradient gel electrophoresis experiments. Simulations start at $t=0$ with a homogeneous protein, either native or denatured, distributed uniformly at the top of gel (left in the figures). Simulated patterns computed for 150 min of electrophoretic migration for the dissociation and unfolding reactions starting with native trimers (center) and for the refolding and association reactions starting with denatured proteins (right).

The reconstitution constant k_{rec} at low urea concentration is significantly higher than predicted from linear extrapolation of log k_{rec} to 0 M urea in Fig. 4. This discrepancy is not unexpected because assembly of the trimers from folded monomers is not a monomolecular reaction as was assumed for simplicity in the fit to the transport equations. Hence, a non-linear dependence of log k_{rec} on urea concentration is reasonable.

As a test of the model, patterns for the protein distribution in the urea gradient gels were simulated by profiles at times 0 and 150 min, for 'experiments' in which native and denatured C subunit were applied to the gels. Both simulations, shown

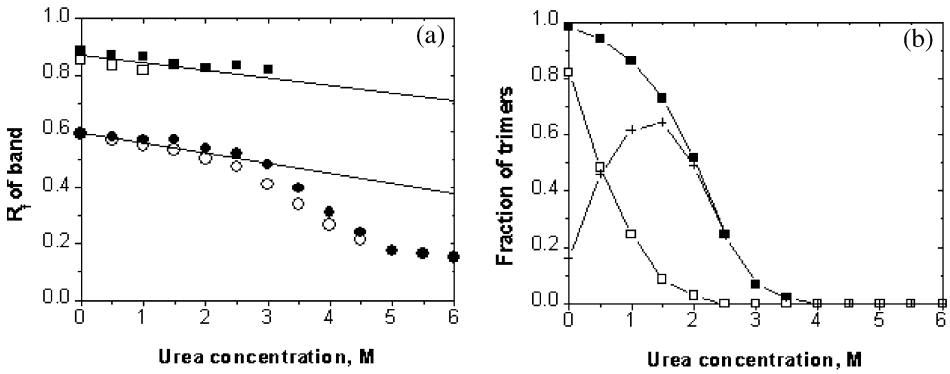

Fig. 6. Unfolding and dissociation transitions derived from simulation profiles in Fig. 5. (a) R_f at the band maximum showing urea promoted changes for intact trimers (□, ■), and for the reversible unfolding of monomers (○,●). Open symbols refer to the association and refolding reactions and solid symbols designate the dissociation and unfolding reactions. (b) Fraction of trimers obtained by integrating the appropriate band in the simulations shown in Fig. 5; the process starting with native protein is designated by (■) and for the denatured protein by (□). The crosses (+) represent the differences in the fraction of trimers in the simulations for experiments with native and denatured protein.

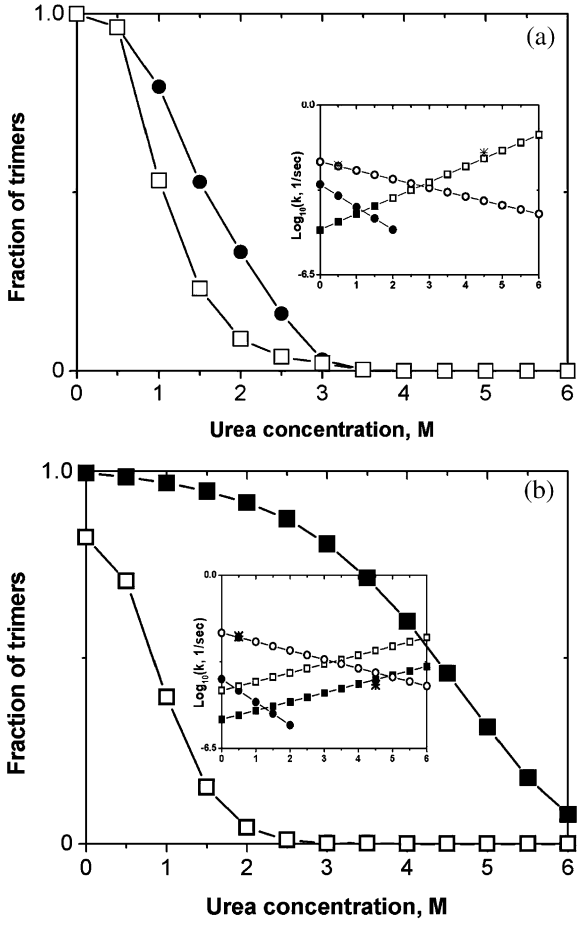

Fig. 7. of active site ligands, pyrophosphate (Pp_i) and carbamoyl phosphate (Cbmp), on the dissociation of C trimers and unfolding of the polypeptide chains. Calculations of the fraction of protein as trimers and various rate constants were based on simulations with parameters yielding the best fit to the experimental gel patterns. (a) Fraction of trimers in the presence of 5 mM pyrophosphate for experiment starting with native protein (●) and with denatured protein (□). Insert shows kinetic rate constants for dissociation, k_{dis} (■); for reconstitution, k_{rec} (●); for unfolding, k_{unf} (□); and for refolding, k_{ref} (○). Asterisks indicate the values of rate constants estimated from circular dichroism measurements (unpublished). (b) Fraction of trimers determined from experiments with 5 mM carbamoyl phosphate (Cbmp) with ■ representing the experiment with native protein and □ for experiment with denatured protein; the corresponding rate constants are shown in the insert.

in Fig. 5, started with the Gaussian band positioned at $R_f = 0$ and run for 150 min (not in real time) generating profiles very similar to those obtained in actual experiments (compare with Fig. 1). The total amount of protein in each generated profile remained the same because the model does not include any mechanism of protein dissipation like aggregation or chemical degradation.

Following the same approach as used for processing of the experimental gels, the R_f values for the N_3 and M bands as well as the fraction of trimers at various urea concentrations were derived from the simulated gel patterns and plotted vs. urea concentration in Fig. 6a,b. Excellent agreement between the calculations based on the model and the values deduced from the experimental data, shown in Fig. 2, provides evidence supporting the validity of the model and the choice of parameters.

3.6. Effect of active site ligands on the denaturation of trimers

The utility of the method is illustrated with additional studies demonstrating the effect of active sites ligands on the stability of the C trimers. As seen by the simulation in Fig. 7a, the addition of pyrophosphate causes a marked destabilization of the trimers. In contrast, the substrate, carbamoyl phosphate, enhances the stability of the trimer substantially, as seen in Fig. 7b.

These results are in complete accord with earlier studies [24] based on the formation of hybrid molecules when dissimilar C trimers were incubated together in the presence of various ligands. The slow step in these exchange experiments was the dissociation of the trimers into monomers, which could then associate at random to give hybrid trimers. Similarly, the urea gradient gel electrophoresis experiments demonstrate that the active site ligands affect the dissociation process rather than the unfolding of the monomers to disorganized polypeptide chains.

Acknowledgments

We thank Dr Ying R. Yang for constructive discussions. This research was supported by

National Institute of General Medical Sciences research grant GM 12159.

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