Denaturation and Self-Association of Apolipoprotein A-I Investigated by Electrophoretic Techniques

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ABSTRACT: Purified human apolipoprotein A-I (apoA-I), when run across a transverse urea gradient at alkaline pH, gives a complex pattern characterized by a number of parallel sigmoidal curves, in which the transition between high- and low-mobility forms, *i.e.* from folded to unfolded structure, occurs between 1.1 and 3.2 M urea. Size differences appear to be the major cause of this isomerism. When migrated across a wide pH range in the presence of varying amounts of urea to display its titration curve, apoA-I is resolved into two pairs of bands, running parallel in the neutral to basic pH region while merging at acidic pH; such a finding does not correlate with a differential exposure of His residues, as shown by diethyl pyrocarbonate titration. Ferguson plot analysis, confirmed by cross-linking experiments, demonstrates a gradual shift from higher to lower mass aggregates as the urea concentration is raised; the monomeric form undergoes denaturation by swelling to an ≈50% larger hydrodynamic volume than in its native state. At alkaline pH, where apoA-I exists as aggregated species, disaggregation and unfolding appear to happen at once, the larger aggregates being less stable than the smaller ones. At acidic pH, apoA-I does not form aggregates and has little secondary structure; unfolding is then a progressive rather than a cooperative process.

Human apoA-I,[1] a single polypeptide chain of 243 amino acids, is the main component of high-density lipoproteins (HDLs). ApoA-I is among the most extensively characterized apolipoproteins, but in contrast to the LDL receptor-binding domain of apoE (Wilson *et al.*, 1991), it has never been amenable to crystallization; hence, its three-dimensional structure is not known in detail. Information about its arrangement in solution is therefore primarily investigated by indirect means.

Amphipathic helices have been recognized as the building blocks of apoA-I structure (Segrest *et al.*, 1974; Brasseur *et al.*, 1990). Considerable attention has then been devoted to apoA-I topology within its physiological lipid environment and to HDL assembly in either spherical or discoidal forms. To this aim, the orientations of the amphipathic helices toward one another and with respect to the lipid moieties have been assessed by combining spectrophotometric techniques with the use of proteinases as conformational and topological probes, mostly on homogeneous reconstituted HDL samples (Jonas *et al.*, 1989, 1990; Dalton & Swaney, 1993). The shape of lipid-free apoA-I in solution was investigated by ultracentrifugal, viscometric, and fluorescence polarization techniques; the molecule was suggested to be an elongated ellipsoid in its monomeric form and to aggregate through the end-to-end association of side-by-side dimers (Barbeau *et al.*, 1979).

In the experiments that prompted the present investigation, apoA-I denaturation was assessed, according to Creighton (1979), by migration across a transverse urea gradient. In this technique, the unfolding of a protein molecule correlates with the reduction in the electrophoretic mobility. The transition from the high- to low-mobility form occurs over a defined interval of chaotrope concentrations, which results in a sigmoidal protein migration course.

While comparing the stability of wild-type and mutant apoA-I toward denaturing agents, we observed a peculiar behavior of purified apoA-I upon unfolding in the presence of urea; a number of components could be resolved by electrophoresis over a *ca.* 2 M urea concentration range. The present report investigates the underlying mechanisms, by evaluating shifts in the extent of self-association as well as changes in surface charge of the protein as a function of chaotrope concentration. This investigation only relies on electrophoretic techniques. In fact, the M_r of apoA-I (28 kDa) and its aggregation number (≤ 6) are far too low for a clear-cut assessment of aggregation phenomena by light scattering experiments. Through electrophoretic techniques, the problem may be approached in two ways. According to Ferguson (1964), untreated samples are run in gels of different polyacrylamide concentrations (*T*%); the larger the molecular size of the protein (or protein aggregate), the more pronounced the decrease in mobility effected by increased sieving from the polyacrylamide matrix. Alternatively, protein assemblies may be stabilized prior to testing by chemical cross-linking (Swaney & O'Brien, 1978).

MATERIALS AND METHODS

Purification of Apolipoprotein A-I. ApoA-I was prepared as previously described (Calabresi *et al.*, 1994) by gel

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[1] Abbreviations: apoA-I and apoE, apolipoprotein A-I and E, respectively; *C*%, relative concentration of the cross-linker in a PAA gel; CA, carrier ampholytes for isoelectric focusing; DEPC, diethyl pyrocarbonate; HDL and LDL, high- and low-density lipoprotein, respectively; IgG, immunoglobulin; IPG, immobilized pH gradient; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; *T*%, total monomer concentration in a PAA gel.

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filtration of the delipidated HDL fraction collected by ultracentrifugation ($d = 1.063-1.21$ g/mL) on a Sephacryl S-300HR column, in the presence of 4 M guanidine hydrochloride. The relative abundance of the 0, −1, and −2 forms (Sprecher *et al.*, 1984) in the purified sample was 40, 30, and 15%, respectively (from the densitometric evaluation with NIH Image, release 1.52, of the protein pattern in Figure 2).

Standard Protocol for Electrophoresis across a Transverse Urea Gradient. ApoA-I denaturation curves were evaluated by running the purified protein across 0 to 8 M urea gradients (Creighton, 1979; Goldenberg & Creighton, 1984). The polyacrylamide (PAA) matrix had a concentration of *T*5 *C*4; the buffer was 70 mM Tris/27 mM tricine (pH 8.6) added with 5.5 mM calcium lactate, according to Monthony *et al.* (1978). Gels were cast against GelBond PAG foils (FMC, Rockville, MD), in a 0.5 mm thick cassette (18-1013-74 from LKB Pharmacia, Uppsala, Sweden); sample application trenches were shaped with 170 × 2.5 mm^2 strips of embossing tape glued to the glass plate. A 4 mL concentration plateau of the 8 M urea mix was poured first, followed by an 8 mL urea gradient, delivered from a two-chamber mixer (18-1019-87, from Pharmacia), and by a further concentration plateau from the no-urea solution. Gels were run in horizontal electrophoresis chambers (Multiphor, from LKB Pharmacia); each electrodic vessel contained 300 mL of fresh buffer. Three layers (150 × 250 mm^2) of 3MM chromatographic paper (Whatman, Maidstone, U.K.) were used for the wicks. The run lasted 90 min, at 15 °C, under a constant applied field of 400 V. Purified apoA-I (100−150 μg), from 1 mg/mL protein solutions, was usually applied per gel. Staining was with 0.3% Coomassie in 30% v/v ethanol/10% v/v acetic acid or with silver nitrate according to Rabilloud *et al.* (1992).

Electrophoresis Runs at Various pHs across a Transverse Urea Gradient. The pH dependence of the denaturation behavior of apoA-I was investigated by running the purified protein across 0 to 8 M urea gradients, in the presence of different buffers, according to Goldenberg (1989). Gels were cast with a *T*5 *C*4 PAA matrix as above; the composition of the various separation media, voltage, and duration of the runs as well as the mode of sample application are listed below: buffer at pH 3.8 = 30 mM β-alanine/20 mM lactic acid, 2 h at 500 V, sample migration from the anodic edge; buffer at pH 4.8 = 80 mM γ-aminobutyric acid/20 mM acetic acid, 150 min at 500 V, sample migration from the anodic edge; buffer at pH 6.1 = 30 mM histidine/30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 2 h at 400 V, sample migration from the cathodic edge; buffer at pH 7.4 = 43 mM imidazole/35 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 90 min at 400 V, sample migration from the cathodic edge; buffer at pH 8.7 = 50 mM Tris/25 mM boric acid, 1 h at 400 V, sample migration from the cathodic edge.

Cross-linked apoA-I (see below) was run at pH 3.8 and 8.7 either by itself or from a parallel trench together with untreated protein under the same experimental conditions.

Isoelectric Focusing across a Stepwise Urea Gradient. An immobilized pH gradient (IPG) (Bjellqvist *et al.*, 1982) covering the range of 4−10 with a nonlinear course (Gianazza *et al.*, 1985) was polymerized, washed, and dried according to standard protocols (Righetti, 1990). Different sections of the gel were then reswollen in solutions containing 0.5% w/v CA for isoelectric focusing and increasing concentrations of urea (2, 2.25, 2.5, 2.75, and 3 M). Purified apoA-I (20 μg per 10 mm wide lane, loaded at the cathode) was run at 15 °C, overnight at 50 V/cm and then for 2 h at 200 V/cm. Protein bands were detected according to Righetti and Drysdale (1974).

Electrophoresis in PAA Gels with Various %T Values. ApoA-I was migrated at pH 8.6 (Monthony *et al.*, 1978) in gels with various PAA concentrations (*T*4, *T*6, *T*8, *T*10, and *T*12). The experiments were repeated in the absence of urea and in the presence of increasing concentrations of the chaotrope, from 1.25 to 4 M. The whole set of data was then analyzed in Ferguson plot format (Ferguson, 1964; Retamal & Babul, 1988). The log of protein mobility was plotted against the corresponding gel concentrations, and the slope of the resulting straight line was computed. The molecular size of the test sample, inversely proportional to the slope above, was estimated by comparing its mobility to that of a series of standards. α-Lactoglobulin, carbonic anhydrase, ovalbumin, and bovine serum albumin could be used as references at alkaline pH since they do not unfold below 3 M urea (unpublished data). They do so, however, at pH ≤4.8 (unpublished data); hence, the Ferguson approach could not be used for assessing apoA-I aggregation in acidic media.

Electrophoresis across a Transverse pH Gradient (Rosengren et al., 1977). Molds 2117-801 from LKB were used to polymerize *T*5 *C*4 polyacrylamide gels, containing 2% w/v CA in the pH range of 3.5−10 (LKB Pharmacia), glutamic and aspartic acid (5 mM each), lysine and arginine, and urea in different concentrations (2, 2.25, 2.5, 2.75, and 3 M). A pH gradient was established by running the gels, without sample, for 2 h under a constant applied power of 7 W per slab, and at a temperature of 8 °C. After 75 μg of purified apoA-I was loaded in the trench (spanning each slab from anode to cathode along the direction of the electric field in the first step of the experiment), the migration of the protein was started at a right angle. The run lasted 30 min at 400 V. The gel slabs were backed with silanized glass plates; the protein stain was according to Blakesley and Boezi (1977). More details on the protocol of this kind of experiments, which may be described as depicting the pH mobility, or electrophoretic titration, curve of a protein, may be found in Righetti and Gianazza (1981). In this setup, the protein curve crosses the application trench where pH = p*I*; where pH ≠ p*I*, the distance migrated is proportional point by point to the varying charge of the molecule. In turn, the charge variation across a given pH range is proportional to the number of dissociating groups whose p*K* is comprised within that pH interval. For multimeric proteins and protein complexes, the amino acids which only have influence on the electrophoretic mobility are those on the surface of the protein [within 6 nm of the outer shell (Shervet & Lakshimi, 1973)].

Histidine Reaction with DEPC. ApoA-I was reacted with DEPC (Jacobsen, 1972; Miles, 1977) to give final ratios of the reagent to histidine side chains of 1/5, 2.5/5, 5/5, and 50/5. The treated samples were analyzed by IEF on a 4 to 10 IPG reswollen in 8 M urea, in parallel with untreated apoA-I.

ApoA-I Cross-Linking. Cross-linking was performed by addition of 1 part dimethylsuberimidate (DMS) solution, 10 mg/mL in 0.3 M triethanolamine, to 10 parts protein solution

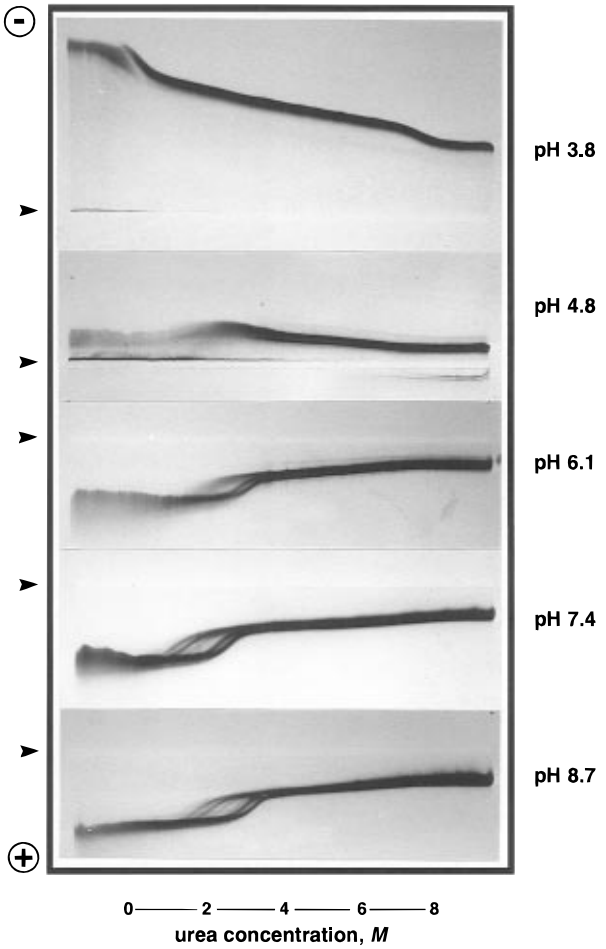

FIGURE 1: Migration of purified apoA-I across a transverse 0 to 8 M urea gradient, in gels with various pHs. The arrows on the left indicate the migration origin.

and incubation for 2 h at room temperature (Swaney & O'Brien, 1978), followed by extensive dialysis. The reaction was performed either in plain buffer or in the presence of urea, at a final concentration between 1.25 and 4 M. Reacted samples were analyzed by SDS−PAGE on 4 to 10% *T* PAA gradients, using the Tris/tricine buffer system in Schägger and von Jagow (1987). The gels were scanned at 627 nm using an UltroScan XL laser densitometer (Pharmacia Biothech, Uppsala, Sweden).

RESULTS

Migration Patterns of Purified ApoA-I across a Urea Gradient, in Gels with Various pHs. ApoA-I susceptibility to denaturation was investigated as a function of pH in an electrophoretic setup according to Creighton (1979); Figure 1 is a composite of the resulting curves. In the shift from pH 4.8 to 6.1, apoA-I moves from below to above its p*I*; hence, the direction of migration is inverted.

Effective Urea Concentrations. The chaotrope range across which a change in mobility occurs, approximately between 1 and 3 M, does not vary with pH.

Migration Course. Where pH > p*I*, the shape of the curves is sigmoidal. The mobility of the folded structure is twice as large as that for the unfolded state. The width of the band is also 3−4 times broader in the former than in the latter case. The very same pattern is observed for sample concentrations of 0.01, 0.1, and 1 mg/mL (not shown). Peculiar mobility shifts are observed at acidic pH. At pH 4.8, much of the protein precipitates in the application pocket at low urea concentrations. The migrating protein displays a lower mobility than observed in 3 M urea; from the latter concentration on, apoA-I mobility decreases linearly without steep steps. At pH 3.8, the precipitation at the application point is reduced both as percentage of the loaded amount and as a range of the unfavorable urea concentration (0−0.5 M). A steep step is observed around 0.5 M urea, together with splitting of the curve. The mobility toward the cathode then decreases linearly up to 8 M urea.

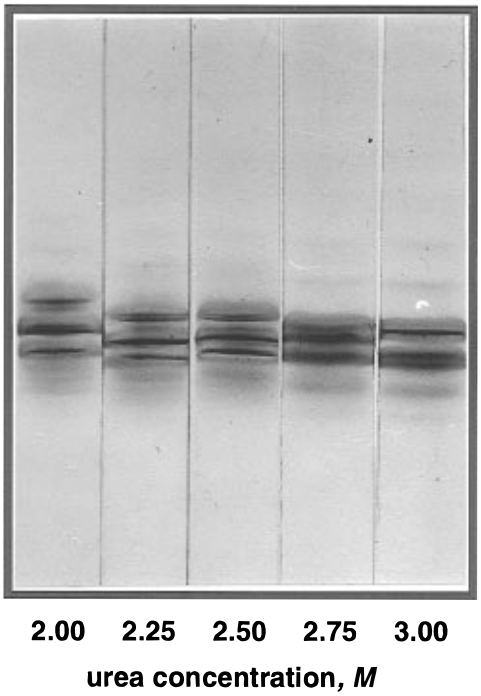

FIGURE 2: Isoelectric pattern of apoA-I as a function of urea concentration, in the 2−3 M range. Anode at the bottom, closeup over a pH span of ca. 2 pH units centered at pH 5.5.

Number of Resolved Components. Just one trace is detected at pH 3.8, and two bands running side by side are detected at pH 4.8. At pH 6.1 and higher, four major and two minor components are stained between 1 and 3 M urea, giving rise to several parallel transition steps.

Reversibility of the Denaturation Process. At least at alkaline pH, where it was tested, pretreatment of the sample with 8 M urea results in an electrophoretic pattern identical to that for the sample loaded in plain buffer, *i.e.* no change is observed either for the concentration range where transition occurs or for the number of resolved components (not shown), indicating that the denaturation process is fully reversible.

Effect of the Surface Charge on the Banding Multiplicity of ApoA-I in 2−3 M Urea. The contribution of charge to apoA-I heterogeneity as detected in the 2−3 M urea concentration range was investigated with three approaches.

Isoelectric Focusing in the Presence of Various Urea Concentrations. The effect of urea concentrations on the p*I* of apoA-I is shown by the experiment in Figure 2, where the protein is run on a 4 to 10 IPG reswollen in a stepwise gradient between 2 and 3 M urea. Over this concentration range, the charge shift is minimal; still, the baricenter among the most prominent forms shifts from higher to lower p*I* values as the urea concentration is raised.

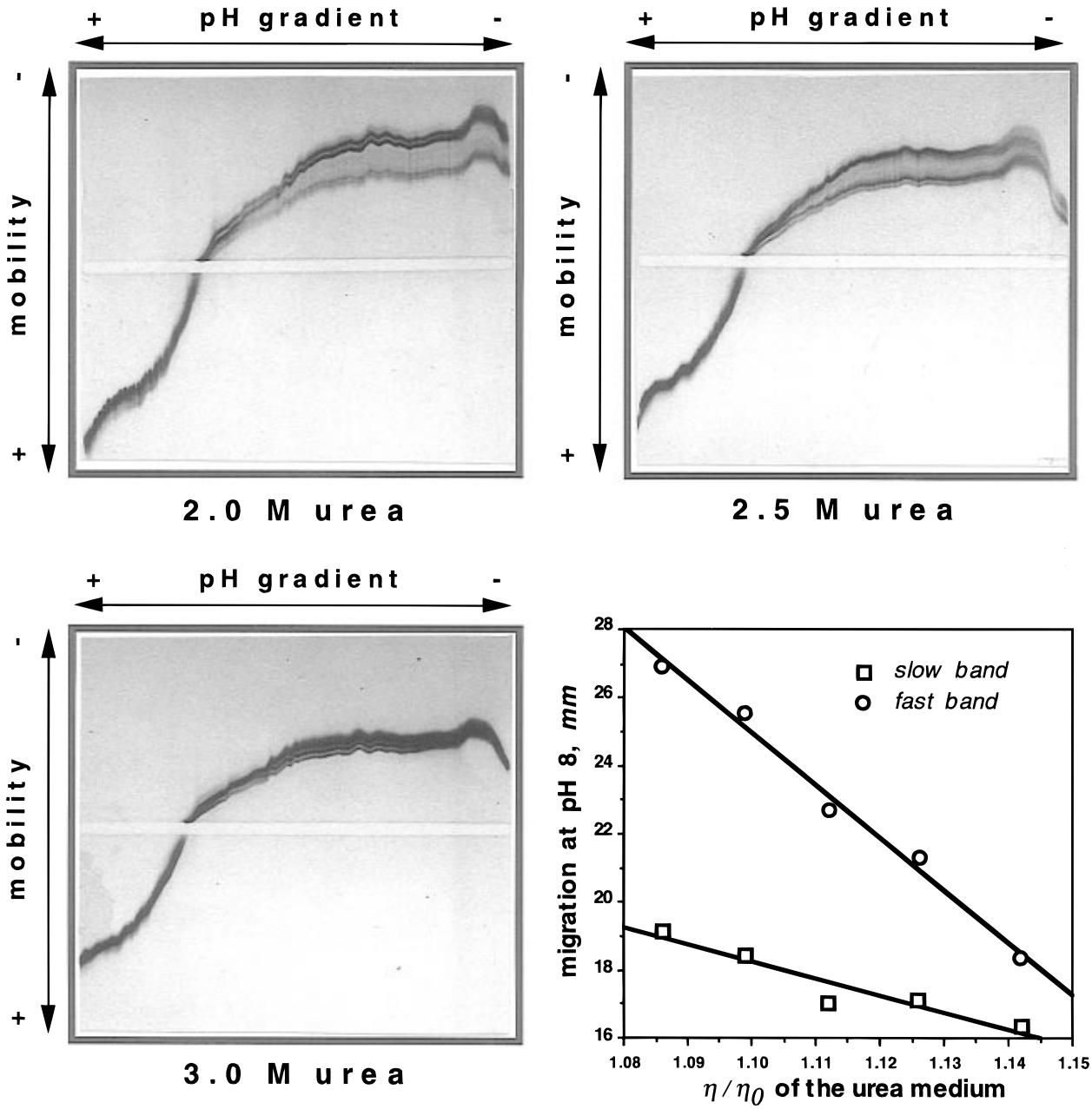

FIGURE 3: Electrophoretic titration curve of apoA-I as a function of urea concentration (2, 2.5, and 3 M). The lower right panel shows the relationship between the migration distance at pH 8.0 and the viscosity of the medium.

pH−Mobility Curves in the Presence of Various Urea Concentrations. Figure 3 compares the migration of apoA-I across pH 3.5 to 10 gradients in the presence of increasing amounts of urea, between 2 and 3 M. At low urea concentrations, two pairs of curves can be detected, which cross the application trench in close proximity. The two pairs run parallel to one another above pH 7 and merge at acidic pH, thus showing different slopes at intermediate pH (5.5−7). As the urea concentration is raised, the migrated distance decreases 3 times more rapidly for the faster pair of curves than for the slower one; the decrease in mobility of both pairs strictly relates to the increase in viscosity of the gel medium due to the higher urea concentration (lower right panel). At the highest urea concentration used, *i.e.* 3 M, the two pairs move close together, and the different bands can be hardly differentiated.

Histidine Titration in the Presence of Various Urea Concentrations. The peculiar behavior of apoA-I as a function of pH could be consequent to alterations of net surface charge (Righetti *et al.*, 1978; Gianazza & Arosio, 1980), as a result of a differential exposure of the five His residues in the apoA-I molecule (Brewer *et al.*, 1978). However, identical p*I* shifts were observed when His residues were progressively modified by titration with DEPC in the presence of varying urea concentrations (2−4 M, not shown).

Effect of the Size on the Banding Multiplicity of ApoA-I in 2−3 M Urea. The contribution of size to apoA-I heterogeneity in the 2−3 M urea concentration range was investigated with three approaches.

Ferguson Plot Analysis. Figure 4 compares the size of the apoA-I species present at various urea concentrations, as evaluated through Ferguson plot analysis at alkaline pH. In plain buffer (no-urea panel), apoA-I displays a broad band due to the presence of multiple molecular species from native monomers up to aggregates with size above a pentamer. In 2 M urea, only two apoA-I species are detectable, with

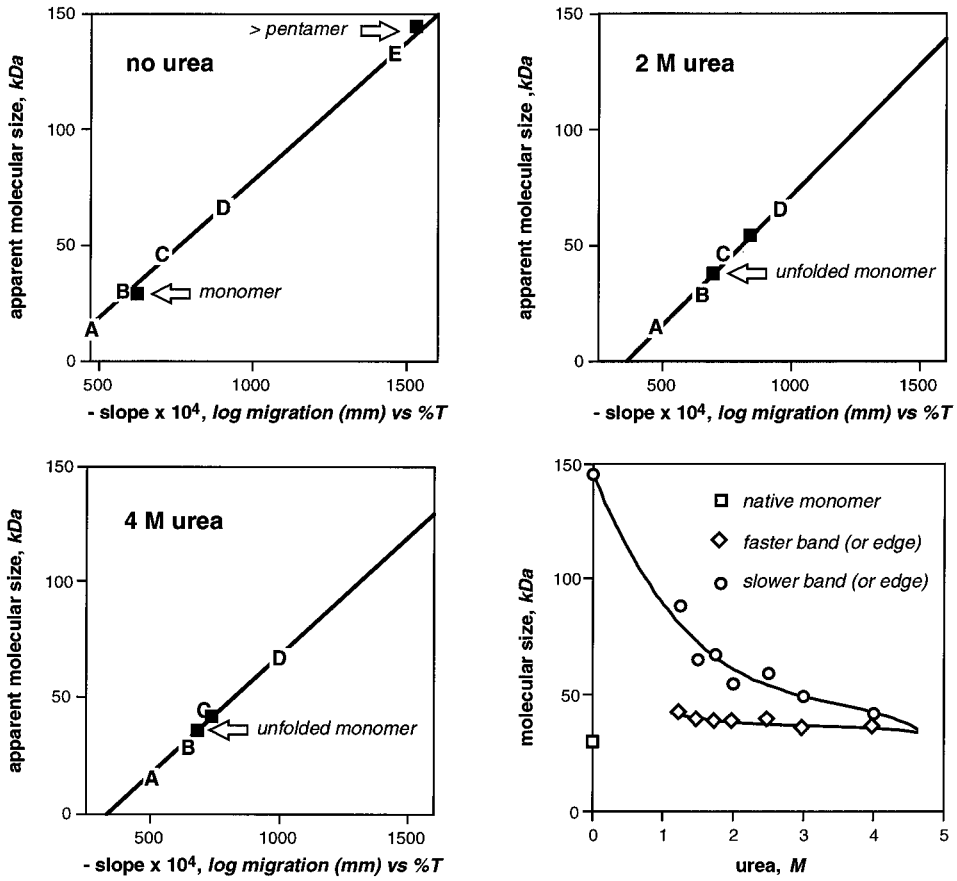

FIGURE 4: Evaluation of the apparent molecular size of apoA-I species according to Ferguson, under native conditions (no urea panel) and in the presence of either 2 or 4 M urea. Molecular mass standards: A = α-lactoglobulin, B = carbonic anhydrase, C = ovalbumin, D = bovine serum albumin monomer, and E = bovine serum albumin dimer. The lower right panel shows the size dispersity of apoA-I as a function of the urea concentration.

apparent molecular sizes of ~66 and ~35 kDa. The latter is the only apoA-I form detected in 4 M urea and should correspond to the unfolded monomer. The lower right panel illustrates the shift from larger to smaller aggregates as a function of urea concentration.

Cross-Linking of ApoA-I in the Presence of Various Urea Concentrations. To identify apoA-I species intermediate between monomer and pentamer which were not resolved by zonal electrophoresis in gels with low urea concentrations, apoA-I was cross-linked with DMS in the presence of 0−3 M urea, and the molecular species were separated by SDS−PAGE. With increasing urea concentrations, the monomer becomes the predominat species, whereas oligomers larger than a dimer almost disappear at a urea concentration above 1.5 M (Figure 5).

Denaturation Behavior of Cross-Linked apoA-I. The denaturation of cross-linked apoA-I was analyzed by electrophoresis across transverse urea gradients at different pHs (Figure 6A), and across a pH gradient in the presence of 2 and 3 M urea (Figure 6B). In contrast to native apoA-I, which shows banding multiplicity in 2−3 M urea at alkaline pH and single banding at acidic pH (Figure 1), cross-linked apoA-I displays multiple bands at pH 3.8 and a single transition step at pH 8.7 (Figure 6A). Under the latter condition, only a minor change in mobility upon denaturation is observed, owing to the rigidity of the reacted molecules. An opposite behavior of cross-linked *versus* untreated apoA-I was also shown by pH−mobility curves (Figure 6B). All together, these experiments demonstrate that the heterogeneity of apoA-I at intermediate urea concentrations is mostly ascribed to size rather than to charge differences.

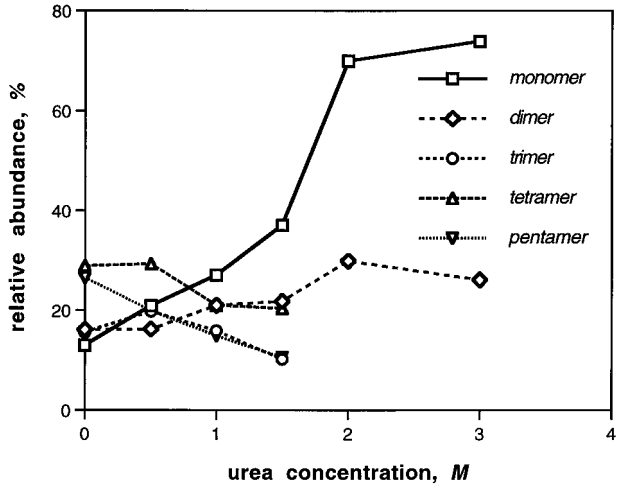

FIGURE 5: Distribution of apoA-I species as a function of the urea concentration, calculated from the densitometry of the SDS−PAGE pattern of a cross-linked sample.

DISCUSSION

Literature Data on Multiple Transitions in Denaturation Curves. The presence of a number of parallel tracings in the transition region of a denaturation curve has been discussed at the theoretical level by Goldenberg and Creighton (1984). Such a finding is taken to imply that the sample

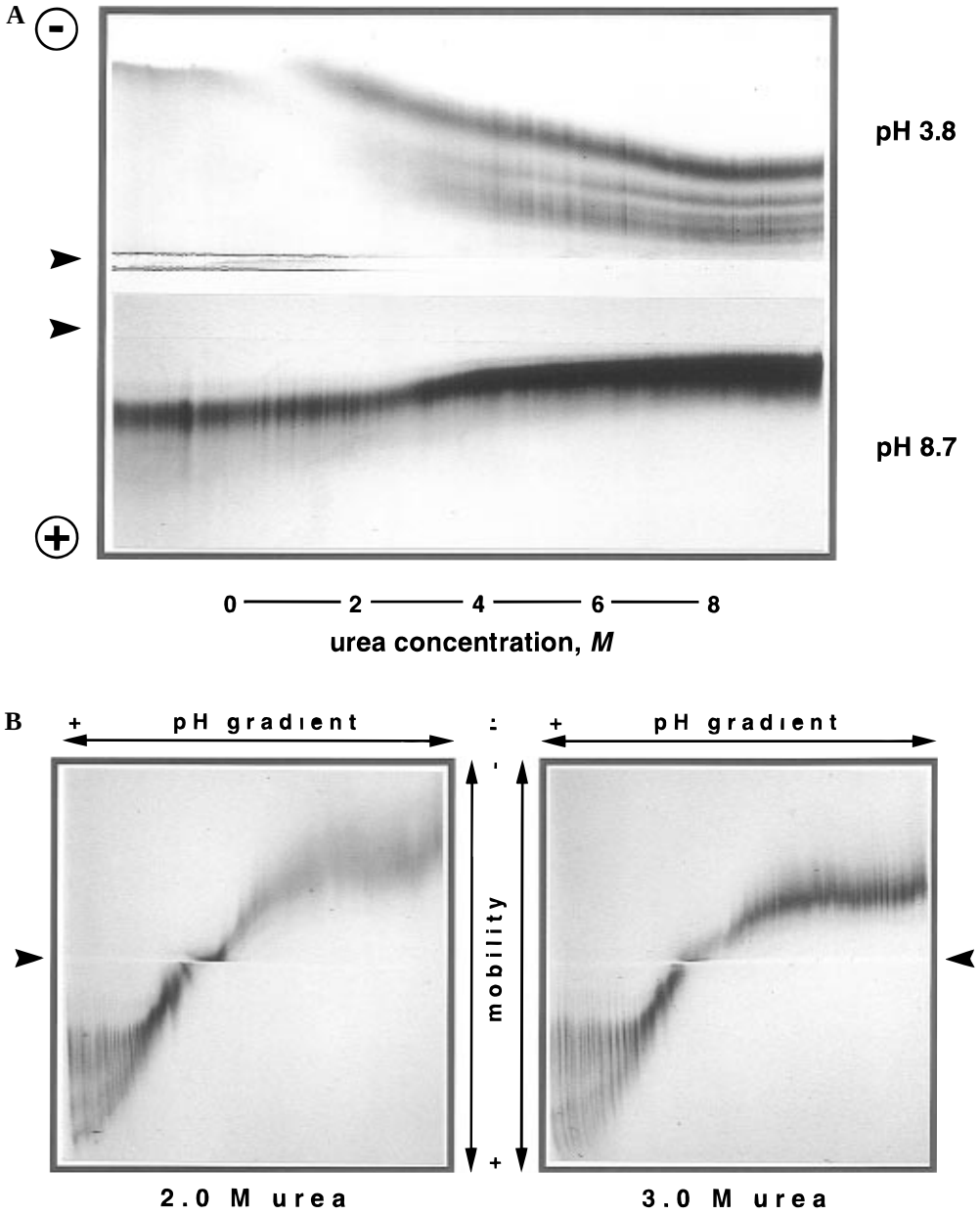

FIGURE 6: (A) Migration of cross-linked apoA-I across a transverse 0 to 8 M urea gradient, in gels with various pHs (pH 3.8, top, and pH 8.7, bottom). (B) Electrophoretic titration curve of cross-linked apoA-I in the presence of either 2 (left) or 3 M urea (right). The arrows on the sides indicate the migration origin.

is heterogeneous; the different species unfold at different urea concentrations, yet all of them have the same native and unfolded mobilities. Few examples of such a behavior can be found in the literature (Science Citation Index, 1979 to June 1996). One case deals with the folding of *Escherichia coli* thioredoxin into two compact forms, which differ by a *cis*- to *trans*-proline isomerization and display different stability to urea denaturation (Langsetmo *et al.*, 1989). In other instances, the effective urea concentration differentiates mutant variants of either staphylococcal nuclease (Creighton & Shortle, 1994; Carra & Privalov, 1995) or transthyretin (Jenne *et al.*, 1996). Somewhat different is the case of the reduced *versus* the oxidized forms of Cys-39 dihydrofolate reductase, for which the overall folding/unfolding pathway changes significantly (Villafranca *et al.*, 1987). None of the mechanisms above seems to apply to apoA-I behavior.

ApoA-I Heterogeneity. In the present study, we show that human apoA-I displays banding multiplicity at all tested pHs above p*I* when run across a urea gradient gel; the resolved species might differ in either surface charge or size. Indeed, a purified apoA-I preparation, as that analyzed in the reported experiments, is known to be heterogeneous in both respects.

(a) Charge. Mature apoA-I is microheterogeneous, with a number of species differing by a single charge unit; −1 and −2 isoforms (Sprecher *et al.*, 1984) arise through a metabolic modification that removes amide groups from Asn (or Gln) residues (Ghiselli *et al.*, 1985). The process begins on the circulating molecule, as the −1 isoform represents more than traces in freshly drawn blood (Anderson & Anderson, 1977; Hughes *et al.*, 1992). Some further modification of the protein may occur during purification due to nonenzymatic deamidation of Asn residues, with loss of ammonia (Harding, 1985).

Surface charge heterogeneity *per se* would not be sufficient to generate the multiple-transition pattern observed upon urea gradient electrophoresis. When analyzed by the same

technique, a number of proteins known to resolve into a variety of isoforms upon isoelectric focusing [including the well-characterized eight glycoforms of α_1-antitrypsin (Yu *et al.*, 1995)] do not split in the transition region. Moreover, the apoA-I charge isoforms separated by isoelectric focusing (Figure 2) are less than the transition steps resolved in Figure 1.

(b) Size. Purified human apoA-I is known to exist in solution in a concentration-dependent state of self-association (Calabresi *et al.*, 1994). In the present experiments, in order to easily detect the protein bands with Coomassie staining, the sample of purified apoA-I was prepared at a concentration of 1 mg/mL, which exceeds the threshold for apoA-I self-association. No changes in the overall pattern were observed when diluting up to 100 times the protein solution, and resorting to silver staining. This finding, however, does not rule out the occurrence of aggregation phenomena during the electrophoretic run. On the contrary, it is known that, if applied in a low-molarity buffer, the sample proteins tend to concentrate while entering the PAA matrix at the onset of an electrophoretic separation. This results from the combined effect of two factors: the Kolraush law on the relationships between concentration and mobility and the sieving effect of the matrix toward high-M_r components.

The multiple apoA-I species resolved by urea gradient electrophoresis may be explained in terms of size differences. The assessment of retardation coefficients as a function of PAA concentrations according to Ferguson (Figure 4), as well as the evaluation of the molecular size of the resolved aggregates after cross-linking of apoA-I in the presence of various amounts of urea (Figure 5), consistently points to a monomer ↔ polymer equilibrium being shifted to the left by increasing chaotrope concentrations. Also, the results from pH−mobility curves may be explained in terms of size differences; the lower right panel of Figure 3 demonstrates a differential sieving for the two resolved doublets as a function of medium viscosity in the presence of increasing concentrations of urea.

Effect of pH. A further demonstration that the multiple individual transitions observed in urea gradient electrophoresis are due to the presence of different molecular species in the apoA-I sample is derived from the changes in the denaturation pattern as a function of pH. When the buffer pH is lowered to 3.8, with partial loss of secondary and tertiary structure (Calabresi *et al.*, 1994), the typical sigmoidal transition between folded and unfolded structure disappears and a progressive slowing of the protein migration is observed (Figure 1). This feature should correspond to a gradual instead of to an abrupt break of the residual intramolecular interactions that shape the three-dimensional conformation of the protein. As a result, the transition proceeds in a noncooperative way. Most likely, the weakness of the underlying secondary structure and the electrostatic repulsion from positive charges also prevent the self-association of apoA-I at low pH. This is made evident by the different pattern in the acidic range of the pH−mobility curves of the native (Figure 3) and cross-linked apoA-I samples (Figure 6), where the presence of stable covalent bonds prevents the dissociation of the supramolecular aggregates.

Dissociation versus Unfolding. The addition of urea to the medium appears to consistently bring about two distinct effects on the apoA-I state: the dissociation of supramolecular aggregates and the unfolding of the polypeptide chain. The signs of either phenomenon on the electrophoretic pattern may be questioned. A decrease in the electrophoretic mobility can only reflect some increase in hydrodynamic volume; hence, slowing is a marker of protein unfolding. This assumption is experimentally confirmed by the coincidence between the urea concentration range over which the change in mobility is observed in electrophoresis and that over which spectroscopical changes are observed in CD experiments (L. Calabresi *et al.*, unpublished results). A mark of the dissociation process may be found in another feature of the curves, *i.e.* band width. In the limb of the denaturation curves (Figure 1) generated at urea concentrations lower than those causing unfolding, the width varies, among experiments, as a function of pH and, within a single experiment, as a function of urea concentrations. Moreover, Ferguson plot analysis in the absence of urea, treating as two independent components the faster and slower edges of the single, broad band of apoA-I, distinguishes multiple molecular species corresponding to monomers up to pentamers (Figure 4, lower right panel).

The present results can also provide information on the timing of the two processes: dissociation of higher aggregates into monomers and unfolding. If the loss of higher-order structures would proceed from quaternary assembly down to tertiary folding and to secondary interactions, then the expected pattern would also have distinct, nonoverlapping stages. The higher mobility limb of the curve would become thinner, as the size dispersion of the sample would disappear, and then all the molecules in monomeric form would undergo denaturation at once. While the first of such features is detectable in the actual denaturation pattern, the transitions to the lower mobility form are manifold. When the dissociation of apoA-I oligomers is prevented by chemical cross-linking, the latter feature disappears (Figure 6A). The number of independent transitions observed upon transverse urea gradient electrophoresis (Figure 1) corresponds to the number of oligomers detected after cross-linking (Figure 5). Moreover, the staining intensities of the different individual transition curves (Figure 1) closely agree with the monomer and oligomers proportions (Figure 5).

Chemical cross-linking and Ferguson analysis suggest that the larger oligomeric aggregates appear to dissociate into an observable intermediate before unfolding. This may be inferred from the disappearence of large molecular species at low urea concentrations with two major species remaining at concentrations above 1.5−1.75 M urea. These species correspond to the unfolded monomer and to an apoA-I species with an apparent molecular size of ≈66 kDa (Figure 4). This estimate exceeds the expected value for an unfolded dimer much more than would be compatible with experimental data dispersion. It can be noticed that this supposedly unfolded dimer is only 20% larger than the native form, whereas the unfolded monomer is 50% larger than its folded counterpart. Some overlap exists, as for nature and strength, between intra- and intermolecular noncovalent bonds; hence, the hierarchy from short to long range interactions is not absolute. This may be especially true for apoA-I whose sequence contains several 11-mer or 22-mer repeat units, arranged in α-helices (Segrest *et al.*, 1974); therefore, the interactions between these elements are essentially the same both within a single molecule and within aggregates.

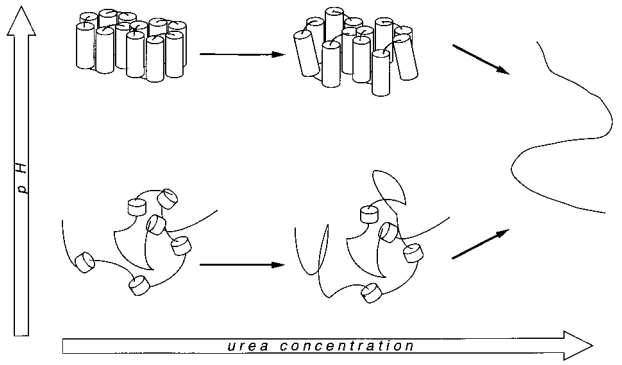

FIGURE 7: Diagram of structural transitions for apoA-I as a function of the pH and the urea concentration.

Concluding Remarks. The behavior of apoA-I when pH > p*I* exemplifies one of the theoretical models of protein denaturation devised by Goldenberg and Creighton (1984); in fact, the present description corresponds to the first such example reported in the literature in which no changes in primary structure exist between the species being compared.

The cartoon of Figure 7 summarizes the main features of the model we propose for apoA-I dissociation and unfolding. At acidic pH, apoA-I has little secondary structure [10% of α-helical content *versus* 50% in a neutral buffer (Calabresi *et al.*, 1994)], and does not form aggregates. A tertiary structure is only loosely defined, as the protein stretches folded in a tridimensional arrangement do not strongly interact with one another. They may thus be differentially influenced by urea, and unfolding is a progressive and not an all-or-none process. At alkaline pH, where apoA-I exists in a self-associated form, disaggregation and unfolding appear to happen at once, the larger assemblies being affected before the smaller ones; the cartoon details the pathway undertaken by the dimeric species.

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