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ARTICLE

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Probing conformation and conformational change in proteins is optimally undertaken in relative mode

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Abstract Hydrodynamic bead modelling has been widely used in attempts to assess the 3D conformation of proteins in solution. Initially, simple models employing only a small number of beads were used, with a considerable degree of success. Latterly, high-resolution bead models based upon atomic coordinates have been developed, and much more sophisticated questions can in principle be addressed. A detailed analysis is presented of the errors involved in the generation of such models and associated prediction of (translational friction) parameters, and in the practical measurement of these parameters for comparison. It is shown that in most cases, for a particle of only moderate asymmetry, the errors are such that it is not feasible to determine, on an absolute basis, which of a range of candidate conformers is the "correct" one. However, when the properties of the candidate conformers can be compared in relation to those of a "paradigm conformer", whose structure in solution, on the basis of external evidence, can be accepted as correct, then errors cancel and very precise comparisons become possible. The generation of 3D bead models (and hence 3D data files) for a range of candidate conformers is a simple matter, using the existing program MacBEADS, further facilitated by a 3D display module (pro Fit).

Keywords Bead modelling · Diffusion · Errors · Friction · Sedimentation

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Introduction

The conformation of proteins in solution in relation to their biological function is a major focus of current study in structural biology. X-ray crystallography (XRC) provides powerful insights into the three-dimensional structure of many proteins, and in some cases yields the same information for conformers formed, for example, in the presence of bound ligands. However, there are two intrinsic limitations to the widespread use of XRC to define a range of solution conformations:

1. The packing energy minimum associated with the formation from individual protein molecules of a 3D crystalline array – often in high concentrations of salts and other compounds – may be associated with a selection rule operating upon a range of conformers co-existent in solution. An example is the recently solved XRC structure for an IgG1 monoclonal antibody (Harris et al. 1998). Here a definitely asymmetric conformation of the protein seen in the crystalline state is unlikely on energetic grounds to correspond to the precise structure in solution of this flexible molecule.
2. The use of XRC is extremely laborious and is thus hardly feasible where the intention is to scan a range of samples and/or conditions. For example, one worker might without difficulty scan the conformation of six or more samples in a day, using both analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). In contrast, many of these samples might not crystallize, even after months of work.

2D NMR can, in principle, be employed to define conformation states of proteins, although it has seldom been employed in this way. It generally requires isotopic labelling of the protein, whose mass concentration needs to approach the millimolar level (i.e. some 10–100 times greater than is required for most solution methods).

Further, in routine modes it only addresses sizes of 20–30 kD or less.

Thus hydrodynamic and scattering methods, which are rapid to use and applicable over a very wide range of molecular mass, are of great potential use in defining conformation and conformational change in proteins or other macromolecular solutes. In this communication we concentrate on the hydrodynamic parameters. We focus our attention on the methods which relate to translational friction. This latter quantity is usually estimated from measurements of either the sedimentation coefficient(s) using the AUC or from the translational diffusion coefficient (D) from DLS. Rotational friction is notoriously difficult to measure, whilst the measurement of intrinsic viscosity requires large quantities of sample and a good deal of perseverance if accurate values are to result. We are additionally well aware of the parallel power of scattering approaches, and indeed consider that the simultaneous application of hydrodynamics and scattering is, where feasible, optimal (see, for example, the study on the bacterial toxin pneumolysin by Gilbert et al. 1999).

In any attempt to define conformation (or changes in same) in terms of measured solution parameters (sedimentation coefficient, diffusion coefficient, among others), one is faced with a simple problem. How does the 3D conformation relate to those parameters? ''Classical'' approaches, in which simple shapes (e.g. ellipsoids) are used in conjunction with a supposed ''hydration'' level to define solute frictional properties, have severe limitations. The use of a ''hydrodynamically equivalent ellipsoid'', especially where a tri-axial (as opposed to rotational) ellipsoid is used (Harding 1989), is elegant and circumvents the ''hydration problem''. However, it yields limited information so far as defining real changes in a 3D protein structure is concerned. Additionally, the strain on experimental technique in acquiring sufficient high-precision data to permit the parameters of a ''hydrodynamically equivalent ellipsoid'' to be computed are formidable (Harding and Rowe 1982). The use of ellipsoids which fit to the mass distribution given by XRC data, as has recently been proposed (Carrasco et al. 2001), cannot be valid. Hydrodynamic parameters are a function of the surface only, where the ''surface'' is defined by the zero shear plane and takes into account solvent interactions. Serious errors will be liable to result if mass distribution ellipsoids are fitted to particles which are highly inhomogenous with respect to local mass density, such as glycoproteins.

A more attractive and widely used approach is to compute directly the hydrodynamic parameters of a postulated structure, and compare these parameters with those actually measured. This involves ''hydrodynamic bead modelling'', in which the 3D structure of the protein (or other particle) being investigated is approximated by a set of spherical beads, of total volume equal to that of the protein and its hydration shell, and distributed in space so as to approximate the actual particle shape. The history of this approach is well documented (Garcia de la Torre and Bloomfield 1981; Garcia de la Torre 1992) and need not be rehearsed further. Particularly with the theoretical work in recent years of Garcia de la Torre, the methodology has advanced to the stage where the frictional properties of a bead model, whether space-filling or surface shell, accord with expected experimental values for friction to within a precision of 1–2% (Carrasco and Garcia de la Torre 1999). Moreover, algorithms have been devised whereby for a protein of known XRC structure a bead model can be constructed automatically from the atomic coordinates, taken from a PDB file (Byron 1997; Garcia de la Torre et al. 2000).

It is now appropriate to attempt to define a general framework for the application of bead modelling to real problems. In this current work we have two principal aims:

1. To consider the extent to which it is feasible, given known constraints of precision in both theory and experimental practice, to distinguish amongst a set of proposed candidate structures for a particle, using hydrodynamic bead modelling.
2. To attempt to define simple ways in which putative changes in conformation can be assessed for plausibility by the construction of bead models for a range of candidate structures proposed on the basis of external criteria.

We focus our attention on the methods which relate to translational friction. An analysis of the factors which influence the accuracy with which the translational friction can be measured shows that there are serious limitations on what can be achieved using hydrodynamic bead modelling in the definition of absolute 3D structure. However, there are real possibilities for distinguishing amongst a range of ''candidate conformers'', the latter being defined relative to a ''paradigm'' conformer whose structure is known with some confidence on the basis of independent criteria.

Materials and methods

Determination of sedimentation coefficients

Analytical ultracentrifugation was performed using a Beckman XL-A analytical ultracentrifuge. Sedimentation coefficients were computed by means of the program Svedberg (Philo 1994).

Theory and results

We focus our attention on two issues which relate to the limitation imposed by the absolute accuracy and relative precision attainable in practical hydrodynamics. If we are examining protein structures whose 3D conformation in solution is not known from other methods:

1. To what extent is it feasible to distinguish on an absolute basis between a range of candidate conformers?

2. If the conformation of the protein can be defined under one set of conditions, on the basis of other techniques, to what extent can changes in conformation relative to that defined species – perhaps caused by ligand binding or other environmental changes – be identified?

In both cases we will in turn need to consider our findings in relation to the level of precision, noted above, with which bead modelling can define measurable parameters.

Can we distinguish on an absolute basis between candidate conformers?

If we are to say that a protein in conformation *i* out of a set of candidate conformers 1. . . *n* is more consistent with hydrodynamic data than one more, or ideally all, of the others, then some measurable parameter of the protein in conformation *i* must be predicted to differ significantly from that measured for the ''excluded'' conformers. We need to understand both how good a prediction we can make of the parameter, using bead modelling, and how accurately we can then actually measure the same. As noted earlier, the accuracy with which the translational friction can be estimated by hydrodynamic bead modelling for a particle of known surface structure is now close to 1–2% (Carrasco and Garcia de la Torre 1999). A problem as yet incompletely solved, however, relates to the complexities introduced into the surface geometry of the protein surface by interaction of surface groups with water. This is often called the ''hydration problem'', but the interactions involved are much more complex than the simple binding of water, and are better described by scaled particle theory (Reiss 1965), as discussed and developed by Chalikian et al. (1996). Progress is currently rapid in this area of protein–solvent interaction (Harding 2001). It may be that in the near future it will become possible to define an ''effective total surface geometry'' for a protein of known 3D structure, by combining work from many methods (NMR, scattering, molecular dynamics, and others) with a definition of the meaning of an effective bound quantity (Rowe 2001). A positive feature is that the percentage error in estimates of friction must, algebraically, be some three times smaller than the error in an effective volume of water bound. Possibly we may soon be able to compute the latter to the order of 3–6%; this would yield an uncertainty in the friction of 1–2%. Combining this error with that arising from the uncertainty in the modelling (also 1–2%, above), we can see that we might, at best and in the near future, predict the absolute translational friction of a macromolecular particle to within 3%, at a modest confidence level.

However, what level of accuracy can be attained in the experimental estimation of translational friction? The simplest method to employ is DLS, provided – and this is important – that the sample under analysis is both rigorously monodisperse and low in overall asymmetry to avoid any angular dependence effects. The translational friction F is then given by the well-known relation:

$$F = RT/D \tag{1}$$

The quantities are in molar terms. Errors in temperature (T) and in the temperature correction factor can be made negligible, at least for work close to 20 °C. It is not difficult, using well-defined samples and accumulating data over an hour or more, to achieve a statistical error in the retrieved parameter D of only a few tenths of 1%. Unfortunately, the run-on-run precision is worse than this, being of the order of 1% (unpublished work from this laboratory). The reasons for this are not clear. Combining errors, using DLS as the experimental probe, will require the candidate conformer to differ by at least 3–4% in its friction as estimated by DLS from the friction estimated for candidate conformers to be excluded.

Is the situation better if we use *s* values, determined in the AUC? The estimation of absolute *s* values is a difficult matter. A range of factors needs to be considered in relation to the accuracy of the finally retrieved parameter(s). These can be listed as follows, with the errors associated with each factor considered first independently and then together.

The use of absorption optics has been assumed. Interference optics ought in principle (Rowe and Harding 1988) to give a higher precision in s using the major algorithms, although at a (probably small) cost in accuracy in terms of greater problems in location of the meniscus position. The latter is required for the use of methods based upon solutions of the Lamm equation or upon use of the time derivative of the data set (below). However, any gain arising from an improved precision in individual data points using interference optics is likely to be trivial given the size of the total data set for the run, which one is fitting globally. Although older methods based upon estimation of ''boundary'' migration do not have the limitation of uncertainty in the meniscus position, they make very inefficient use of the data set, reducing each individual scan to a single number (the ''boundary position''). They thus have little or no use in modern practice.

1. Algorithm used for deriving *s* values from data. It is very difficult to assign a value to the error involved here. The commonly used algorithms (approximate solutions of the Lamm equation; time derivative methods) clearly involve approximation. Simulated ''perfect'' data can only be generated by methods (Claverie et al. 1975) which themselves involve approximation, thus limiting the possibility of an empirical approach. Based upon laboratory experience with a single skilled person analysing (real) data sets by multiple algorithms, we do not find deviations – for well-defined systems – which are above a few tenths of 1% in magnitude. A value of $\pm 0.3\%$ is probably conservative. It is important, however, to ensure that the algorithm used is appropriate to the

system: thus the Svedberg program (Philo 1994) is optimal for lower s values, whilst DC/DT+ (Philo 2000) is generally to be preferred for higher s values.
2. Temperature of the rotor. This is usually the most important single cause of error in s values. Over the range 5–20 °C, s changes on average by around 2.5%/°C, mainly due to changes in solvent viscosity (this factor is itself temperature dependent, and is less in the region close to "room temperature"). The stated accuracy of the temperature for the Beckman XL-A/I is ±0.5 °C. Thus an average uncertainty of ±1.25% in s is indicated for experiments conducted in the range 5–20°C.

Both the absolute accuracy and the constancy of the set temperature have been much discussed on the RASMB site (http://www.bbri.org/rasmb/rasmb.html). A method for checking the absolute temperature has been defined by Liu and Stafford (1995). This method indicated that under ideal conditions, especially of rotor temperature equilibration, the rotor temperature displayed was correct to within ±0.1 °C (at 5 °C), decreasing to ±0.03 °C at 25 °C (Liu and Stafford 1995). This corresponds to an average error in s values of only about ±0.25% for the range stated above. However, as has been discussed by Schuck (2002), there are problems associated with the attainment of set temperature, including the limitations on what can be regarded as a feasible pre-equilibration time in laboratory practice. A small amount of convection associated with the rotor temperature changing during the early part of a run causes methodogical errors in the output from the usual analytical software, which relies upon approximate solutions to the Lamm equation. These latter assume that factors such as temperature do not vary during the run (Schuck 2002). Overall, our present assumption of an average error in s values (over the temperature range 5–20 °C) of ±1.25% is conservative but defensible. Where temperatures close to 20 °C are employed (as is most often the case), then the error will be close to ±0.8%.

3. Speed of rotation of the rotor. For higher speeds (greater than a few thousand rpm) the error is negligible
4. Precision of the cell alignment in the rotor and of the radial values used. The first of these factors may be serious, but is of unknown magnitude. In order to devise a satisfactory experimental test in this area we have re-analysed an extended series of 20 velocity runs carried out by us on a highly monodisperse commercial vaccine sample, average sedimentation coefficient $s = 42$ S (Hepacare, Evans Vaccines, now part of the Powderject Group). An average precision of ±0.37% was found for the standard deviation about a (normalized) mean for repeated runs on multiple preparations of the same (well-defined) sample, where each run comprised three cells with identical contents.
5. Limited precision of values of ρ and η for buffer used in correction factors. These parameters can be measured with considerable accuracy, and their computation from buffer composition is certainly adequate to better than ±0.2%.
6. Pressure effects on s values. Once thought to be insignificant, it has now been shown (Errington et al. 2002) that changes of up to −1.7% can occur with globular proteins, as a result of changes in hydration. Even in the absence of such effects, a change of around −0.4% is predicted in terms of the effects of pressure on solvent density and viscosity. Such effects are minimized by working (where this is feasible) at lower speeds, and by avoiding the use of data near to the cell base, where the hydrostatic pressure is greatest. Under these condition, an error of < -0.3% only should result.
7. Solute concentration effects on s values. For a typical globular protein at 1 mg/mL concentration, an effect of +0.05% is predicted (Errington et al. 2002).
8. Operator to operator consistency. There are subjective elements in the use of the major software packages. The number of scans chosen for analysis, the choices of start scan, of meniscus position, of range in s values and of baseline value for fitting: all these involve individual operator choice. Based upon local work (unpublished data) in which multiple operators have analysed data sets for well-defined systems, we have found that an uncertainty of at least ±0.3% must be allowed for under this heading.

The above estimates of errors contributing to the total uncertainty in estimation of s values are summarized in Table 1. Combining all the non-systematic errors in the conventional manner yields an expected random (±SE) error close to ±1.4% for data over the range 5–20 °C (±1.0% for data close to 20 °C), with a potential systematic error of −0.25% or less. Taking 2SE

Table 1 The individual errors which contribute towards the total uncertainty in the estimation of absolute values for the sedimentation of a well-defined, monodisperse macromolecular solute in the analytical ultracentrifuge. The errors are either systematic as in (6) and (7) or random, as in all other cases. For a full discussion, see text

Source of error in *s*value	Error (%)
1. Algorithms used for deriving s values from data	±0.30
2. Temperature of the rotor	±1.25 (5–20 °C)/ 0.80 (20 °C)
3. Speed of rotation of the rotor	Negligible
4. Precision of the cell alignment in the rotor and of the radial values used	±0.37
5. Limited precision of values of ρ and η for buffer used in correction factors	±0.20
6. Pressure effects on s values	−0.30
7. Solute concentration effects on$s$ values	+0.05
8. Operator to operator consistency	±0.30

as is normal to reflect a high level of confidence, we can say that a measured s value is correct to $\pm 2.8\%$/$\pm 2.0\%$ (random error) for the two temperature regimes stated, with the possibility of up to -0.25% systematic error.

Our overall conclusion has to be that absolute s values cannot be determined experimentally to better than ± 2–3%, even with the most careful working. Of course, the level of consistency which a given operator can achieve on multiple runs carried out in the same manner on the same instrument under the same conditions can be much closer than ± 2–3%. This in no way voids our conclusion concerning the accuracy which can be achieved.

Unfortunately, that is by no means the end of the matter so far as estimating friction via s values is concerned. Unlike the use of the D value, from DLS, there are possible errors in the density increment ($d\rho/dc$) and in the absolute molecular mass (M) to be considered. Where we can take the latter value as error-free, being known from sequence, then it is only the error in the former which needs to be taken into account. With very careful working an additional error of only $\pm 1\%$ is entailed, arising from uncertainty in absolute solute concentration measurements. This assumes that the $d\rho/dc$ term has been very carefully measured at the operating temperature. Considering this error to be random in nature, we combine with our estimate of $\pm 2.8\%$ in s (above) to arrive at a final accuracy of ± 3–4% for experimentally determined s values. Under many conditions this accuracy would be worse.

We must now ask whether these levels of accuracy in measurements of translational friction ($\pm 1\%$ for D values, $\pm 4\%$ for s values) are likely to suffice for distinguishing between alternative conformations in real examples. We consider one clearly defined example, the myosin S-1 fragment, for which a range of conformers are believed to exist and which has been studied in some detail by hydrodynamics (Bayliss et al. 1999; Errington et al. 1999). Rotation of the ''lever arm'' around the head region is probably a larger motion than is found in the majority of supposed conformational changes which might be studied in proteins. Yet the maximal change in translational friction predicted for the transition from the pre-power-stroke state (ADP bound) to the rigor state (ADP release) is -3.7% (Bayliss et al. 1999; Errington et al. 1999). The actual change observed in s value is rather less than this, probably due to a distribution of conformers being present (Bayliss et al. 1999). Similar levels of change in friction have been observed in other, differently regulated, myosin-based motility systems (Stafford et al. 2001).

It is clear that if one merely started out with a preparation of myosin S-1, and a range of models deemed to be equi-probable on other criteria, one could not hope to distinguish which model was actually applicable on the basis of an s value. This is because the errors in estimation of that s value would be of the same order of magnitude as the predicted difference between the candidate conformers (3–4%) and the absolute error in the predicted s value for each of them ($\pm 3\%$).

Might one fare better using D from DLS to estimate the friction? Clearly, the errors in the experimentally determined friction are much lower (1% against 4%). However, given that the accuracy with which friction can be predicted ($\pm 3\%$ at best, see above) is of the same order as the range of s values expected for the candidate conformers (3–4%), one can only conclude that even infinite accuracy in measurement of F would not suffice.

Yet, as noted, the differences in friction between the conformers thought to exist in myosin S-1 are large; they are almost certainly larger than would be found in most cases under study for globular or near-globular protein systems. We are obliged to conclude that identification of solution conformation by the study of the translation friction of a range of closely related possible candidate structures is in most cases not feasible.

Can we characterize changes in solution structure using hydrodynamics?

It requires little thought to see that the above limitations to the determination of absolute conformation should not apply if relative changes only are considered. We are now considering precision, as opposed to accuracy. So far as experimental work is concerned, a brief inspection of the list (above) of the errors associated with the determination of s values shows that for samples being compared within the same rotor (but in different cells) almost all errors are in common, and hence cancel. A final precision of at least $\pm 0.3\%$ is easily attainable. In the case of difference sedimentation (Obefelder et al. 1985) with two samples in different sectors of the same cell, even greater precision can be attained ($\sim 0.1\%$ or even better). Interestingly, there is no gain so far as the use of DLS is concerned, the precision in comparing samples being no better than the accuracy of determining D for a given, single sample. Changes in the predicted friction when computed on a relative basis will also be minimized. It is unlikely that there will be much change in the errors in computation of friction or in the level of solvent interaction associated with a conformational change, and hence these factors too will cancel out.

Thus, if we confine our studies of conformational states to the attempted identification of candidate states whose hydrodynamic parameters are defined relative to those of a paradigm state of known conformation, then we see that the AUC offers us a remarkably high degree of precision ($\sim 0.1\%$ or even less). In this context, the finding of Bayliss et al. (1999) of an experimental decrease in the s value of myosin S-1 of -3.3%, as compared to -3.7% expected for populations being 100% in the two defined states, can legitimately be interpreted. The pre-power-strike state seems to be incompletely populated: a postulate for which there is independent evidence (Bayliss et al. 1999). The rigor state is in this

case therefore the paradigm state. Another example can be taken from the study of the effects of mutational change in the C-terminus of TMADH (Ertegruhl et al. 1998; Errington et al. 1999). Here small changes in *s* value relative to the rc-WT state can be identified via the "conformation spectra", even though the range of *s* values estimated lies within the limits imposed by the absolute accuracy as defined above.

Discussion

Our analysis shows clearly that there are limits to what can currently be achieved in the assessment of the structure of a biological macromolecule in solution, using hydrodynamic methods in conjunction with hydrodynamic modelling. The fundamental problem is this: hydrodynamic modelling predicts the most usable parameter (friction) with an accuracy of only around $\pm 3\%$ at the very best. This latter estimate assumes that the true surface of the hydrated particle is known with some certainty. Yet the total range of friction expected for a range of plausible candidate structures for a particle of only modest asymmetry can be only around 3–4%. Even neglecting the actual experimental error in determination of the friction parameter (around 1% for DLS, around 3–4% for sedimentation), one cannot hope to distinguish one candidate structure from another given the limits on the levels of accuracy and precision noted.

This does not, of course, detract from the utility of hydrodynamic (bead) modelling in answering related but different questions. Suppose we have a model for a newly isolated oligomeric structure: can we say that that model is plausible, given some knowledge of its hydrodynamic parameters? This is a reasonable question to ask, and very many valid examples could be quoted of this approach (see Garcia de le Torre 2003). From our own studies we could cite the assessment of a structure for pneumolysin suggested by electron microscopy (Morgan et al. 1993, 1994) and of the structures, again suggested by electron microscopy, of newly isolated forms of 14S dynein, compared with that of 22S dynein (Tharia et al. 1997). Alternatively, might one legitimately enquire as to whether structures whose predicted parameters lie well outside of the range envisaged above (± 3–4% in friction) could be considered? Clearly, our strictures do not apply in such cases, provided that one bears in mind the limitations imposed by the errors identified. We recall again the work of Bayliss et al. (1999), where certain effectors (e.g. aluminium fluoride) were found to have a relatively extreme effect on myosin S1 conformation. Equally, the recognition of the 10S (as compared to the 6S) form of myosin (Byron 1997) posed few problems, given the gross difference between these two states.

The conclusion has to be that the use of the hydrodynamic bead modelling approach, especially in relation to structures generated from XRC coordinates, is most generally valid and powerful when used in relative mode. In this case, a paradigm state is selected, for which the 3D coordinates are available, and for which there is (preferably) additional evidence from alternative techniques to confirm the applicability of that structure in solution. The remarkably high precision ($< 0.1\%$) with which relative *s* values can be measured in the AUC then becomes a definitive tool in assessing the relative plausibility of selected candidate conformers.

The only barrier to work of this nature is a simple one: given that one does not – almost by definition – have a set of 3D coordinates for a whole range of candidate conformers, for which XRC data are unlikely to exist. So, on what basis can one set up bead models in order to have a file to submit to HYDROPRO (Garcia de la Torre et al. 2000) or other program? To this end we have over several years made use of the program MacBEADS (authored by D.A. Thomas and available, together with the instruction manual, from ftp://alpha.bbri.org/rasmb/rasmb_homepage.html). The instruction manual additionally gives details of a newly developed interface between MacBEADS and the commercial software package pro Fit (Zurich Software). MacBEADS, in addition to making provision for ab initio bead modelling, can import 3D coordinate data (e.g. from a pdb file). The new interface enables almost any sensible modification to the 3D bead model of the paradigm state to be effected in a simple visual manner, with the production of a file of coordinates suitable for submission to a program such as HYDROPRO (Garcia de la Torre et al. 2000).

The testing of hypotheses concerning changes in conformation from a paradigm structure to a candidate conformer or conformers is thus made simple. The assumption which we have made, that factors such as solvation will cancel when one does such a comparison, cannot be rigorously demonstrated, but seems very likely to be true within working limits. A final note of caution must however be sounded. The approach tests hypotheses but it does not finally confirm structures. The "uniqueness problem" will always be there. The need for the additional confirmation of hypotheses using parallel techniques cannot be circumvented.

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