A comparison of mass spectrometry based hydrogen deuterium exchange methods for probing the cyclophilin A cyclosporin complex

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ABSTRACT

Direct infusion electrospray ionisation mass spectrometry (DI-ESI-MS) techniques provide an increasingly popular route to determine quantitative information on protein–protein and protein–ligand interactions. When combined with hydrogen deuterium exchange (HDX), details on protein stability and complex conformation can be obtained; however, complexes retained by ESI-MS are not always representative of those in solution and care must be taken in interpreting gas phase results. Zhu et al. [1] and Powell and Fitzgerald [2] have outlined LC–MS based techniques to probe the solution phase properties of the protein–ligand system in question. We here have taken the well characterised soluble immunophilin protein cyclophilin A, and examined it in complex with its endogenous ligand cyclosporin A. This ligand is widely used as an immunosuppressant following organ transplant, and the complex provides a basis for drug discovery efforts. We have used direct infusion, coupled with HDX, gas phase HDX and also the LC-HDX techniques PLIMSTEX and SUPREX. Results from each of these four HDX methodologies are presented here and discussed critically. From our direct infusion we find that there are 2 observable hydrogen populations in the protein, a very fast exchanging population, and a slower group. The exchange rate of both is lowered in the presence of the ligand. For PLIMSTEX we find a K_d for ligand binding of 321 ± 128 nM, which is within one order of magnitude of values previously reported. SUPREX under a variety of conditions provides a range of K_d values, but when we average these for experimental error we obtain a K_d of 7.11 ± 0.29 nM which agrees well with measurements from other studies including via SUPREX. Finally gas phase HDX of the native complex shows more than 3 distinct populations of exchangeable hydrogens, for both the apo- and the holo protein consistent with an unfolding and refolding of the protein in the gas phase. The different techniques are compared with respect to the advantages and disadvantages they bring to the study of this protein–ligand system.

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1. Introduction

Recent years have witnessed the development of a plethora of techniques which seek to combine HDX and mass spectrometry to probe protein properties [3–9]. The marrying of these two techniques is extremely fortuitous, since exchange of a hydrogen for a deuterium atom results in a mass increase of one. HDX-MS approaches provide a picture of conformational change, which when coupled with data from fragmentation techniques such as enzymatic digestion [10] or dissociation techniques [11] can be used to study protein dynamics with respect to ligand binding [12].

To study the dynamics of larger proteins, detection of conformers and/or intermediate conformations is desirable. Kaltashov

and co-workers have performed extensive studies, measuring deuterium loss during refolding of pseudo-wild type cellular retinoic acid binding protein I (CRABP I), a 15 kDa protein, by monitoring exchange rates [13]. They also studied the unfolding dynamics of the β-sheets in CRABP I and established the presence of a bimodal distribution identifying two discrete exchange populations and hence defining two distinguishable conformations [14]. In the early 1990s the pioneering work of Loo and Smith and Chait established a link between solution phase protein conformation and charge state distributions [3,15]. Chait's contribution employing solution phase HDX [3]. McLafferty and co-workers [6] used gas phase HDX to infer a relationship between gas phase protein conformation, and known solution phase conformation(s). In a comparison between ion mobility mass spectrometry and gas phase HDX for the well characterised protein ubiquitin, Freitas et al. [16] made an important finding. As the charge on the protein increases so too does the recorded collision cross-section [17]. However, Freitas noted that higher charge states exhibited less deuterium uptake, attributable to the requirement of the relay mechanism of Beauchamp and co-
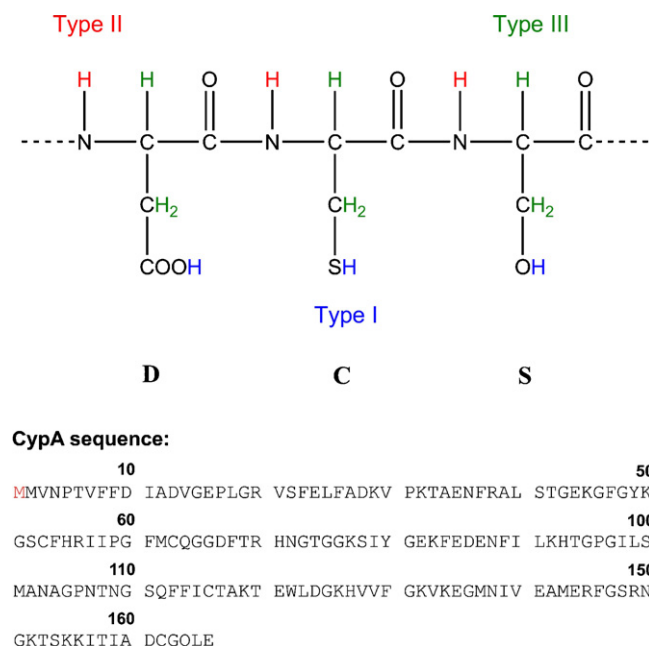

Fig. 1. Types of hydrogens found on polypeptides, along with the sequence of cyclophilin A used in these experiments. For an explanation of the different types see text. CypA has a total of 1245 exchangeable hydrogens: 125 side chains (Type I), 160 amide backbone (Type II) and 952 unexchangeable Type III.

workers [18] that a proton donor and acceptor are <5 Å from each other. This work showed that care must be taken in interpreting gas phase HDX data, particularly of proteins electrosprayed from solutions where they are likely to be denatured. In this manuscript, we present work employing native ESI-MS, incubation of the protein in buffered solutions and the use of mass spectrometry to report on the extent of exchange with and without ligand present.

One of the disadvantages of performing DI-ESI after HDX is that the exchanged deuteriums will exchange out as the analysis proceeds. Within peptide structures there are three types of hydrogen atoms found (Fig. 1) each with differing exchange behaviours:

Type I: Located on side chain functional groups these undergo rapid forward and exchange out at rates that are dependent on the solution conditions (pH, and temperature).
Type II: Amide backbone hydrogen exchange is very sensitive to solution conditions, and the rate is minimum at a pH range between 2 and 3. Dropping pH and temperature to reduce exchange rates is useful for studying backbone folding and unfolding kinetics.
Type III: Carbon bound hydrogens do not exchange under these conditions and time-scales. For the purposes of mass spectrometry HDX measurements, these can be ignored.

For direct infusion HDX experiments exchange from Type I hydrogens, is extremely rapid, leading invariably to the loss of the isotopic label, with the possible exception of the epsilon-hydrogen of arginine side chains. In general the rates of HDX that can be experimentally observed, will reflect on the conformational mobility, hydrogen bonding strength, and solvent accessibility of hydrogens in protein structure. To study protein–ligand interactions via HDX coupled with mass spectrometry several approaches have been developed which employ chromatographic methods to exploit the differing behaviours of the hydrogens present (Fig. 1).

One such method was developed by Gross and co-workers and first reported in 2003 termed PLIMSTEX (**P**rotein–**L**igand **I**nteractions in **S**olution by **MS**, **T**itration and HD **EX**change) [8].

For affinity quantification the method requires a change to occur in the extent of deuterium exchanged during titration. In a PLIMSTEX experiment the protein of interest is first equilibrated with different concentrations of the ligand in a non-deuterated environment, before HDX is initiated with a deuterated buffer. After reaching (near) steady state conditions, defined by Gross et al. [1,19] as being 'when fast exchangeable hydrogens had reached equilibrium whilst the slow exchangers had not' HDX is quenched by lowering temperature to 0 °C and pH to 2.5, giving an end point for the HDX reaction. The solution is loaded on a guard column for a desalting and wash step which back exchanges the side chain deuterons of the immobilised protein. Elution with an organic gradient into the MS for analysis reveals the deuterium uptake due to HDX of amide backbone hydrogens, reflecting the conformational state of the protein prior to quenching. A plot of the mass difference between the deuterated and the non-deuterated protein *versus* the total ligand concentration results in a PLIMSTEX curve. These curves describe the degree of protection due to ligand binding and thereby can reveal the induced change(s) in protein conformation [1].

Another method utilising HDX-MS to probe protein–ligand behaviour is SUPREX. '**S**tability of **U**npurified **P**roteins from **R**ates of H/D **EX**change'. This technique was first reported by Fitzgerald and co-workers in 2002 [20] and has been applied to a number of systems. SUPREX measures the stability (i.e. the standard free energy of protein folding (ΔG_f)) of a protein or a protein–ligand system by denaturation with a chemical denaturant utilising the reactions of globally protected amide protons during HDX. Generally ΔG_f reflects the stability of a protein in its 'native' conformation and therefore differs in misfolded proteins or protein–ligand complexes. In the first instance, a misfolded protein is generally less stable than its 'native' conformation [21] whereas ligand binding has a stabilising effect [22]. Once ΔG_f values have been determined, K_d (dissociation constant for the complex) and m-values can be calculated for the protein–ligand complex [2]. The m-value is the denaturant dependence on the free energy change between two states, an increase in an m-value on ligand binding indicates that the ligand has protected part of the protein from solvent [2].

This paper describes work using different HDX strategies on the immunophilin cyclophilin A (CypA) and the complex it forms with its endogenous ligand cyclosporin A (CsA). Four different approaches are taken and the merits of each critically compared.

CypA is a soluble protein from the cytosolic peptidyl prolyl isomerase (PPIase) family, called immunophilins, its primary sequence is shown in Fig. 1. The members of this family including cyclophilins (Cyp), FK506 binding proteins (FKBPs) and parvulins are enzymes involved in the regulation of protein folding and transport and are often found as part of much larger proteins such as nuclear pore complexes [23–25]. There are four different members of the Cyp family termed A–D, of which CypA is examined here. CyPA is known to bind the ligand cyclosporin A (CsA) [26,27–30] a natural inhibitor of the immunological activity of this protein [31] produced as a metabolite by the fungus *Tolypocladium inflatum*. As a consequence of strong interaction with CypA [27–30], CsA is now a widely used immunosuppressant drug. It is principally administered post-organ transplant to reduce the activity of the patient's immune system decreasing the risk of organ rejection.

Wang et al. [32] have already previously reported MALDI SUPREX measurements to compare purified CypA/CypA:CsA with endogenous CypA (over expressed in lung tumour tissue lysate) with CsA added. Their findings, demonstrated the dissociation constant of purified protein is twice that of the unpurified, although both values lie within the range found in other studies. This work provides a useful benchmark for the work herein, where we take a comparative approach to the difference HDX methodologies with the aim of providing an optimised work flow for HDX based evaluation of protein:ligand interactions.

2. Methods and materials

2.1. Protein and ligand preparation

For the expression of CypA in *E. coli* the psw3-003 vector containing the CypA gene (donated by Prof. M. Walkinshaw, University of Edinburgh) was chemically transformed in BL 21 Star [TM] cells (Novagen – Darmstadt, Germany) as previously described [29]. Further details are found in Supplementary data. Once purified, the protein was concentrated to 500 nM and stored on ice at 4 °C for 6–8 weeks.

Cyclosporin was obtained from Novartis (Basel, Switzerland). All chemicals are from Sigma–Aldrich (Dorset UK) unless otherwise specified.

2.2. Direct infusion (DI) HDX protocol

20 μM solutions of CypA and CypA:CsA (1:1) complex were incubated in 10 mM deuterated ammonium acetate (NH₄OAc) (pD 7.2) or deuterated water. The mass shift was monitored over time by DI-ESI using a Q-ToF mass spectrometer (Micromass, UK) and equated to deuterium uptake.

2.3. PLIMSTEX HDX protocol

1 μL of a stock solution of CypA (122.9 μM) was diluted ten fold in 10 mM NH₄OAc (pH 6.8). To this was added 1 μL of CsA solutions at varying concentrations and the protein–ligand complex then incubated at room temperature for 20 min. CsA stock solutions were prepared as multiples of the CypA concentration. Then 76 μL 10 mM deuterated NH₄OAc and 9 μL MeOH were added to yield a final CypA concentration of 1.2 μM. This was incubated for 80 min before quenching with 1 μL 2 M HCl. The quenched sample was loaded onto a C4 guard column (Optimize Technologies, Oregon City, USA) using an *ensemble* of Rheodyne valves (7067, 7125; Rheodyne, Rohnert Park, USA) combined with 2 Jasco PU980 HPLC pumps using buffer A (97% H₂O, 3% CH₃CN; pH 2.5; Fig. S1). The quenched protein sample was eluted with buffer B (96% CH₃CN, 4% H₂O; pH 2.5) directly into the mass spectrometer.

2.4. SUPREX HDX protocol

SUPREX experiments were performed according to the SUPREX protocol reported by Fitzgerald et al. [9,33,34] A series of SUPREX-buffers from 0 to 6 M with different denaturing concentrations were prepared from guanidinium hydrochloride stock solutions along with 10 mM deuterated NH₄OAc (Fisher Scientific – Loughborough, UK). Concentrations were verified using an Abbe-refractometer (Carl Zeiss – Jena, Germany) at RT and 10 mM deuterated NH₄OAc (Fisher Scientific – Loughborough, UK) as blank. Refractive indices were transformed into concentrations according to published methods. In automated SUPREX experiments the final protein concentration was between 4 and 5 μM, depending on the protein–ligand system, and a final volume of 70 μL. Therefore 4 μL protein (60 μM stock) were placed in a 250 μL, 12 mm × 32 mm autosampler vial and sealed with a snap cap. The SUPREX-buffers were pipetted and sealed in identical vials according to their concentration. To study the CypA:CsA complex, *n* + 1 volume equivalents of ligand were present in an additional vial at concentrations between 10- and 20-fold excess to the apo protein, before incubating for 10–420 min, online quenching, desalting and mass spectrometry analysis.

2.5. Mass spectrometry analysis

For DI experiments a Q-TOF Ultima (Waters UK) was employed. Ions were produced by positive nano-electrospray ionisation using a z-spray source, within a spray voltage range of 1.2–1.6 kV and a source temperature of 80 °C. Sample and extractor cone voltages were optimised to maintain a stable signal. Nanospray tips were prepared in-house from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments Inc., Sarasota, FL, USA) using a Flaming/Brown Micropipette puller (Model P-97, Sutter Instrument Co., Novato, USA). Tips were filled with 10–15 μL of sample using gel loader tips (Eppendorf, Hamburg, Germany). CypA was incubated with CsA and samples were analysed at time intervals from 3 min (the shortest practical time to fill a tip and obtain stable spray) to 3 days.

For mass analysis of SUPREX and PLIMSTEX data a Platform II (Micromass, UK) mass spectrometer utilising positive ESI was used. The capillary voltage was 3.5 kV with a cone voltage of 50 V. The source temperature was set to 65 °C. For HPLC analysis an Ultimate 3000 system (Dionex – Sunnyvale, USA) in conjunction with a C4 guard column (Optimize Technologies – Oregon City, USA) was employed. Protein washing and elution was achieved with buffer A (97% H₂O, 3% CH₃CN; pH 2.5) and B (96% CH₃CN, 4% H₂O; pH 2.5), respectively.

Acquired mass spectra were smoothed using a Savitzky–Golay algorithm, baseline corrected and centred (MassLynx 4.0). For DI experiments the data was deconvoluted using MaxEnt, and the deconvoluted masses used to obtain the rates of HDX.

For PLIMSTEX and SUPREX experiments, the peak list was exported to Microsoft Excel where the data was manually deconvoluted so that the protein mass was obtained as average of the individual masses of the different ionic species. For PLIMSTEX experiments, a curve was generated employing Microcal Origin 7.5 by plotting the deuterium uptake *versus* the ligand/protein concentration and fitted with 3 parameters to obtain the dissociation constant as previously described [19]. SUPREX data was plotted and $C_{1/2}$- and free energy values were extracted *via* a four parameter sigmoidal fitting equation and linear fitting of the $C_{1/2}$-values [20], respectively, culminating in K_ds for the protein–ligand complex in question (see Supplementary data).

2.6. Gas phase HDX

20 μM CypA and CypA:CsA in 10 mM ammonium acetate were analysed by nanoESI using a LCQ (Thermo Scientific) with a modified helium inlet line, which incorporates a reservoir for deuterated solvent. A software patch specifying an extended activation time command of 100 s was supplied by Thermo Scientific to increase the time available for gas phase HDX. The capillary temperature was maintained at 200, 125 and 100 °C and deuterium uptake compared accordingly.

3. Results and discussion

3.1. Direct infusion

Fig. 2 shows the mass spectra obtained from the CypA:CsA complex sprayed at a concentration of 20 μM for both protein and ligand from different solvent systems. Each spectrum comprises of a low charge state distribution where a considerable portion of the CypA:CsA complex has been retained into the gas phase. The distribution is centred on the species due to [CypA+9H]⁹⁺ and [CypA+8H]⁸⁺ and their complexes with CsA. Even after 3 min of exposure to the deuterated solvent (data in Fig. 2) significant changes can be observed, the *m/z* for the [CypA:CsA+8H]⁸⁺ species
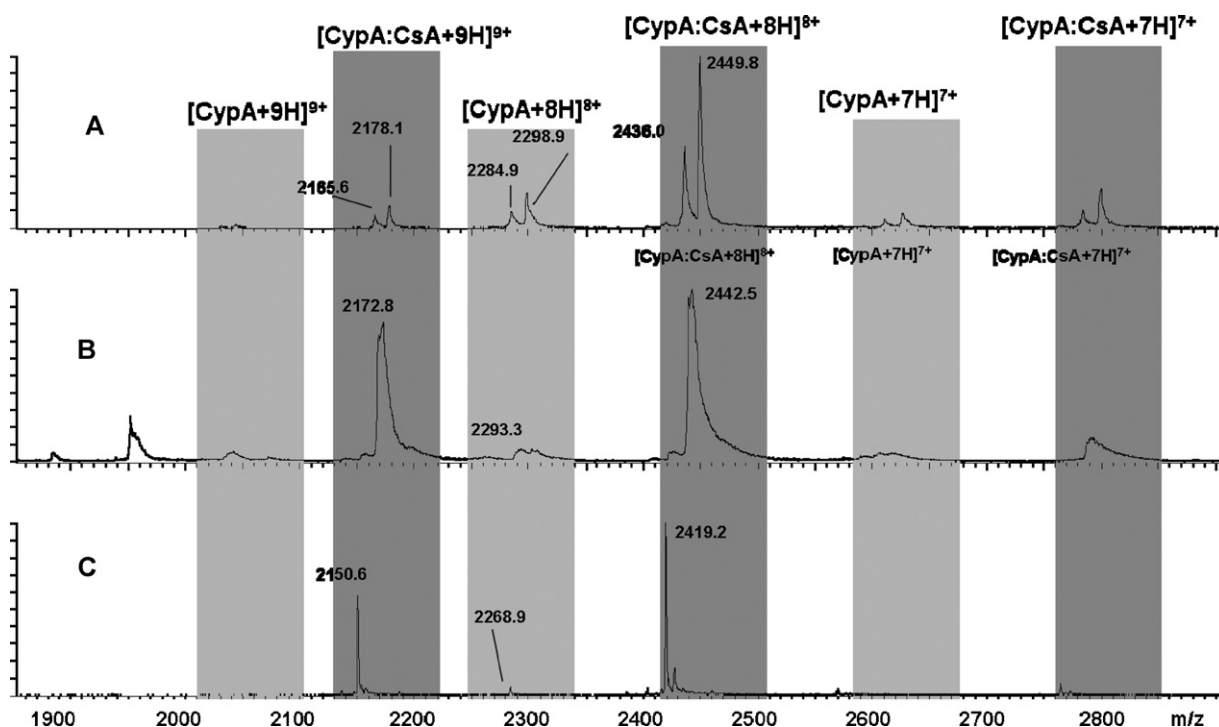
Fig. 2. Mass spectra obtained following nano-ESI of solutions of CypA:CsA (1:1) 3 min after incubation in 10 mM deuterated ammonium acetate (A), deuterated water (B) and deuterium free ammonium acetate (C). The data C shows split peaks for each of the protein species identified. The reason for this is unclear, but the relative intensities of the peaks varied from batch to batch and so were thought to be attributable to a misfolded form of CypA as an artefact of the expression protocol. The SUPREX studies by Wang et al. [32] also found evidence of two isomers in both their purified and tissue lysate CypA. Analysis by MALDI-ToF failed to resolve them once deuterated however and so were regarded as inconsequential. In this study we therefore disregard the second peak and focus on the first.

has shifted from 2419.2 in water to 2442.5 and 2436.0 in deuterated water (B) and deuterated buffer (A), respectively. This represents an uptake of ~178 and ~136 deuteriums out of a total of 285 exchangeable 2 hydrogens in CypA. This uptake was monitored over a period of days and is examined using deconvoluted data in all cases. Table 1 shows the difference in deuterium uptake when the protein is incubated, with and without the ligand, in either water or buffer. The rates for exchange in deuterated water were calculated from 3 min up to 23 min (primary exchange rates) and exchange rates are listed for this primary population in Table 1. The exchange of CsA involved in the complex has been incorporated into the calculated mass shifts. Table 1 also averages the mass increases observed between 0–23 and 23–3100 min (3 days). During the first 23 min, ligand free CypA in water exchanges 39 more hydrogens than with buffer, and a further 20 over the remaining time period. This indicates that there is increased flexibility in water for approximately two thirds of the protein. Conversely, where ligand is present, the uptake appears marginally lower in water than buffer. The exchange rates of bound and unbound CypA in water decrease by approximately 27% during the first time period

but these remain constant in a buffered environment. Immediate exposure of complex to deuterated ammonium acetate appears to increase solvent accessibility relative to deuterated water, i.e. some rapid exchange occurs out with the detectable time period with the first detectable value obtained after approximately 3 min.

These findings support the NMR study of Yan-Hong et al. [35] who report that the rates of exchange for 59 labile hydrogens in the unstructured loop of CypA are so rapid they could not be measured, which could well be the case here, since over the shortest time that we can get a sample that has been incubated with buffer to mass analysis we see exchange that exceeds this (Table 1). The secondary population (defined as being the exchangeable hydrogens between 23 and 3100 min) shows less stability in water (Table 1). This latter observation is probably a result of the reduced ionic strength of water compared to the ammonium acetate solution having a detrimental effect on the stability of the protein fold, and/or the effect of the different pH on exchanges rates in water *versus* the buffered solution.

The complex shows near identical level of exchange when incubated in deuterated water or deuterated buffer (153 and 156

Table 1
Data from direct infusion HDX studies of the CypA:CsA complex.

Protein configuration	Solution	d-Uptake (Da)			k (min^{-1})
		0–23 min	23–3100 min	Total	1° Pop^n
CypA only	Water	204	28	232	7.1×10^{-3}
	Buffered	165	15	180	4.7×10^{-3}
CypA:CsA holo	Water	153	29	182	5.2×10^{-3}
	Buffered	156	21	177	8.7×10^{-3}
CypA:CsA apo	Water	147	30	177	4.4×10^{-3}
	Buffered	158	15	173	1.2×10^{-2}

The total mass increases for CypA over two time ranges are shown along with exchange rate, k calculated from the initial linear region. Data is shown for CypA by itself and when incubated with CsA. For the second set of experiments, the mass spectra contained both holo- and apo protein, and data is given for both forms.
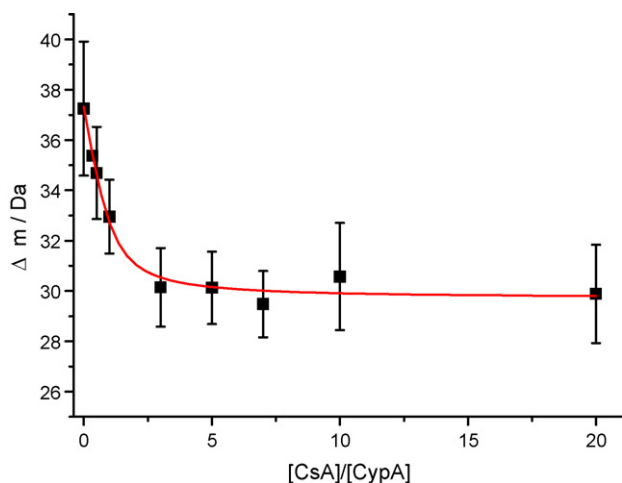
Fig. 3. Manual CypA–CsA PLIMSTEX data, with Δm_{free} 37.32 ± 0.33 Da, $\Delta m_{\text{complex}}$ 29.69 ± 0.25 Da, Δm_s 7.60 Da, K_a $3.12 \times 10^6 \pm 1.24 \times 10^6$ M^{-1} and a K_d of 321 ± 128 nM. Error bars represent standard deviation from five independent experiments.

deuteriums, respectively, in the first 23 min). Equivalent exchange values are seen for unbound CypA in the presence of ligand over this time (158 deuteriums in buffer and 147 in water). Either (a) the unbound CypA is representative of protein released from complex by in source dissociation and/or (b) CypA retains a conformational 'memory' of a bound state in solution. The latter observation would support EX1 behaviour for this protein.

DI coupled with HDX can only report on global changes in the conformation and stability of a protein as a function of ligand binding, and it will not reveal the precise whereabouts of the ligand binding site. But the great potential of this approach is to further our understanding of the dynamics of CsA binding as evidenced by our kinetics data.

3.2. PLIMSTEX

Apo CypA subjected to the PLIMSTEX procedure outlined above gives a Δm_{free} value of 37.32 ± 0.33 Da resulting in a mass of 18,181 Da for deuterated CypA without any ligand present. Titrations up to a 20-fold excess of ligand induce a conformational change or a change in stability of the protein and therefore shows a decrease in deuterium uptake or an increase in protection due to the presence of the ligand. The resulting PLIMSTEX curve for CypA is shown in Fig. 3. Employing the PLIMSTEX fitting procedure as outlined previously [19], values for $\Delta m_{\text{complex}}$ and Δm_i are determined as 29.69 and 7.60 Da, resulting in a K_a 3.12E6 $\pm$ 1.24E6 M^{-1} and a K_d of 321 ± 128 nM. This K_d value is within one order of magnitude of the values previously reported [36]. Critical to PLIMSTEX investigations, is that the investigated protein–ligand system possesses a sufficient degree of protection of amide protons in the presence of the ligand, and/or that there is no significant cooperative conformational alteration of the protein remote from the ligand binding site that would result in an observed mass increase. Data from the direct infusion experiments described above show definite stabilisation of the protein in the presence of the ligand, with respect to HDX, at least for the first 23 min of incubation (Table 1), but the very small Δm_i found here has exacerbated the error in this PLIMSTEX approach (Fig. 3).

3.3. SUPREX

3.3.1. Apo CypA

Apo CypA was subjected to the SUPREX routine and $C_{1/2}$ SUPREX values for incubation times in the range 10–420 min were determined (Table 2, column 2). Fig. 4A illustrates the 10 and 60 min incubation and the induced shift to lower denaturation concentrations at increased exposure. The deuterium level pre-transition was found to be ~25 Da and post-transition ~55 Da, resulting in a 30 Da increase in mass upon denaturation. The programme SPHERE [37] was used to calculate k_{int} for CypA and it was found to be 5.59 s^{-1}. Using this value of k_{int} to plot of $C_{1/2}$ SUPREX *versus* ΔG_f at 22 °C (Fig. 4B) yielded a value for the free energy of protein folding of -32.43 ± 1.88 kJ mol^{-1} (Table 2). The abscissa related errors in Fig. 4B represent the standard error associated with the $C_{1/2}$ non-linear curve fitting whereas the ΔG_f associated error reflects the standard error of the linear fit. The m-value is calculated to be 12.38 ± 3.01 kJ mol^{-1} M^{-1}.

These ESI-SUPREX ΔG_f values compare well with those reported by MALDI-SUPREX and SPROX by Fitzgerald et al. for purified apo CypA -47.28 ± 2.93 kJ mol^{-1} and unpurified apo CypA -39.33 ± 0.84 kJ mol^{-1} [32] and $-19.25\ 1.67$ kJ mol^{-1} [38] for apo CypA. Variation in the absolute ΔG_f values may occur as result of comparing different protein species in different environments. For example purified and unpurified CypA samples have given ΔG_f differences of 7.95 kJ mol^{-1}. Divergent buffer conditions may also affect relative protein stability; in this work as 10 mM NH$_4$OAc was utilised whereas Fitzgerald and co-workers solely employed 20 mM sodium phosphate. SUPREX derived ΔG_f and m-values are not meaningful in themselves, however, relative changes in ΔG_f can be used to calculate comparable dissociation constants for ligand binding.

3.3.2. CypA–MeOH

Part of our aim was to ascertain the benefits of a SUPREX approach for ligand screening so we opted to include methanol as a likely solvent for synthetic ligands in this study. This gave a resultant methanol ratio of 2.3% for the protein–ligand system in the HDX/SUPREX/PLIMSTEX-buffer. Thermodynamic properties of apo CypA were also determined under these conditions. Determined ΔG_f values for apo CypA in the presence of 2.3% MeOH indicate stabilisation, as $C_{1/2}$ SUPREX values are shifted to higher denaturant concentrations by 0.27, 0.39, 0.34 and 0.34 M for incubation times 10, 30, 60 and 120 min, respectively (data not shown). The pre-transition baseline presents an increased deuterium level by about 10 Da for 30 min incubation, or a decreased protection of amide protons suggesting alcohol denaturation of CypA in the presence of methanol. From this methanol exposed protein, the free energy of protein folding is calculated to be -38.74 ± 5.06 kJ mol^{-1} resulting in a stabilisation of -6.32 ± 5.40 kJ mol^{-1} and an m-value of 14.14 ± 4.60 kJ mol^{-1} M^{-1}. The increased m-value in the 2.3% MeOH containing CypA apo sample compared to the pure buffer indicates an increased surface exposure of the protein, most probably a more unfolded state of CypA with a different $C_{1/2}$ SUPREX value.

3.3.3. CypA–CsA

Thermodynamic parameters for CypA–CsA are determined for incubation times from 10 to 420 min. SUPREX curves for a 10 min incubation are shown in Fig. 4C. The free energy of protein folding is here determined to be -44.48 ± 4.35 kJ mol^{-1} resulting in a stabilisation of -12.05 ± 4.73 kJ mol^{-1}, compared to that for apo CypA. Fig. 4C emphasises the strength of the CypA–CsA interaction as the 10 min incubation stabilisation is 1.57 M (listed in Table 2). Average shifts of $C_{1/2}$ SUPREX values are between 1.57 and 2.39 M. The m-value is calculated to 7.11 ± 1.63 kJ mol^{-1} M^{-1} which suggests less surface exposure of CypA in complex with

Table 2
Summary of CypA SUPREX results.

Incubation time (min)	$C_{1/2}$ values		
	Apo CypA	CypA–MeOH	CypA–CsA
10	1.00	1.27	2.57
15	0.68		2.39
30	0.63	1.02	3.02
45	0.54		2.68
60	0.55	0.89	2.64
90	0.48		
120	0.60	0.94	2.53
150	0.51		
180	0.44		2.39
420	0.62		
	Apo CypA	CypA–MeOH	CypA–CsA
ΔG_f (kJ mol^{-1})	-32.43 ± 1.88	-38.74 ± 5.06	-44.48 ± 4.35
$\Delta\Delta G_f$ (kJ mol^{-1})		-6.32 ± 5.40	-12.05 ± 4.73
m (kJ mol^{-1} M^{-1})	12.38 ± 3.01	14.14 ± 4.60	7.11 ± 1.63
K_d (nM)	–	–	596.7 ± 234.7
	Analysis via average m-value (11.21 ± 3.08/kJ mol^{-1} M^{-1})		
	Apo CypA	CypA–MeOH	CypA–CsA
ΔG_f (kJ mol^{-1})	-32.23 ± 0.66	-35.72 ± 0.62	-55.35 ± 0.66
$\Delta\Delta G_f$ (kJ mol^{-1})		-3.26 ± 0.91	-22.89 ± 0.91
K_d (nM)	–	–	7.11 ± 0.29

The $C_{1/2}$ values that were found for the experiments on CypA, CypA with MeOH and CypA in the presence of CsA. Values obtained for ΔG_f, $\Delta\Delta G_f$ and m are given along with the calculated K_d for the CypA:CsA complex. This analysis has been done with separate and averaged m-values as described in the text.
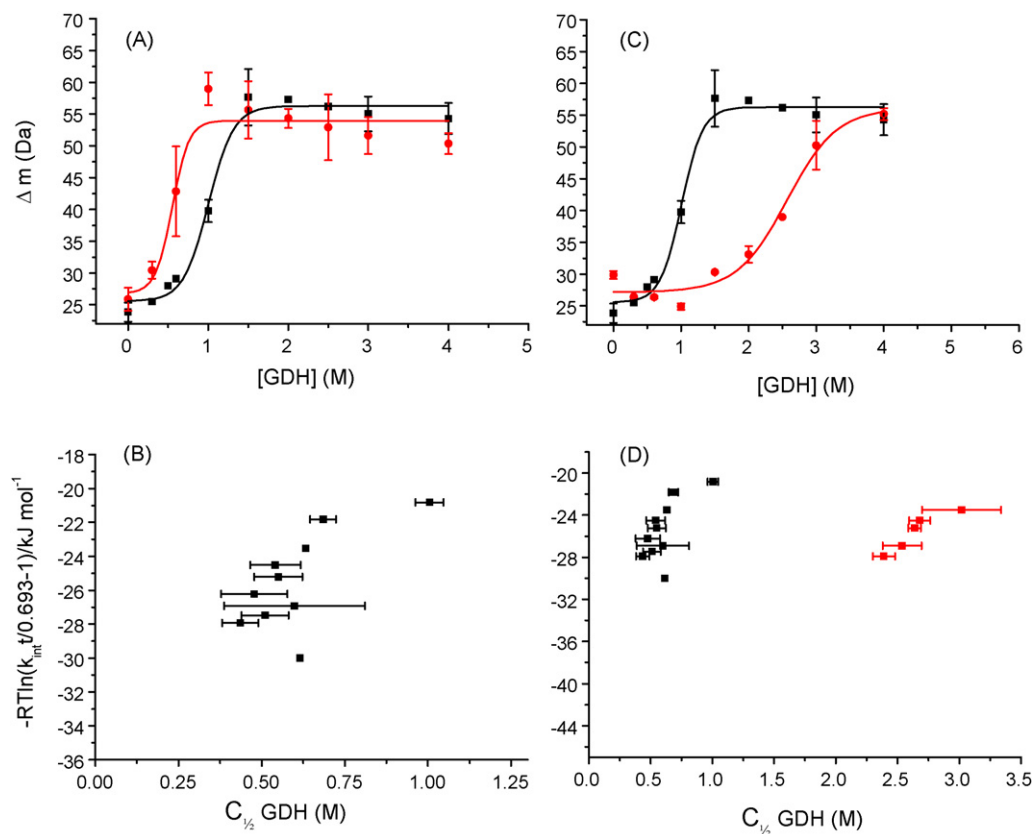

Fig. 4. SUPREX experiments on the CypA and the CypA:CsA complex. (A) Apo CypA, SUPREX curves at 10 (black) and 60 min (red) incubation with $C_{1/2}$ SUPREX values 1.00 and 0.55 M, respectively. Error bars represent standard deviation from two independent experiments. (B) Apo CypA: free energy of protein folding *versus* $C_{1/2}$ SUPREX. ΔG_f is determined to -32.43 ± 1.88 kJ mol^{-1} and m to 12.38 ± 3.01 kJ mol^{-1} M^{-1}. Error bars represent standard error from fitting SUPREX curves. (C) CypA/CsA: SUPREX curves at 10 min incubation for apo CypA (black) and CypA–CsA (red) with $C_{1/2}$ SUPREX values 1.00 and 2.57 M, respectively. Error bars represent standard deviation from two independent experiments. (D) For CypA:CsA free energy of protein folding *versus* $C_{1/2}$ SUPREX. ΔG_f is determined to -32.43 ± 1.88 kJ mol^{-1} and m to 12.38 ± 3.01 kJ mol^{-1} M^{-1} for apo CypA and -44.48 ± 4.35 kJ mol^{-1} and m to 7.11 ± 1.63 kJ mol^{-1} M^{-1} for CypA:CsA (red). Error bars represent standard error from fitting SUPREX curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
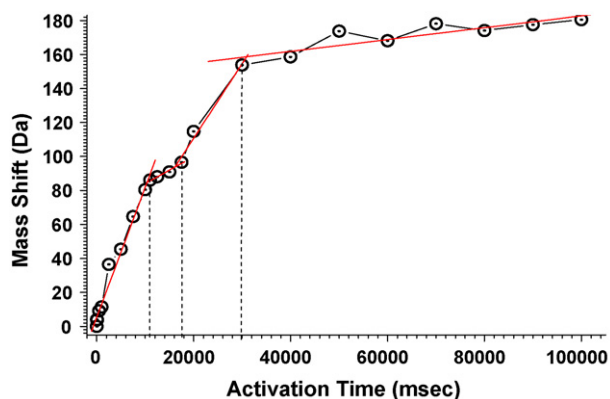
Fig. 5. Deuterium uptake by CypA in the gas phase over 10 s activation time with capillary temperature, 100 °C.

CsA during the unfolding process compared to the free protein. From a SUPREX fit of this data (Fig. 4D) the dissociation constant is determined as 596.7 ± 234.7 nM. The deuterium level of the pre-transition baseline is similar for all CypA and CypA–CsA curves. However, the post-transition baseline is slightly lower for the CypA–CsA complex compared to the free protein, indicating a more compact structure of CypA when bound to CsA. The lower m-value of 7.11 ± 1.63 kJ mol^{-1} M^{-1}, compared to that for free CypA, also indicates less solvent exposure due to a more compact stabilised state. The longest incubation time investigated was 180 min in the presence of CsA, beyond that no CypA could be detected due to degradation.The SUPREX determined K_d of 596.7 ± 234.7 nM is within the same order of magnitude as found above from PLIMS-TEX experiments. Reported values for ΔG_f and $\Delta \Delta G_f$ by Fitzgerald and co-workers are -64.85 ± 2.93 and -58.99 ± 3.34 kJ mol^{-1} and -17.57 ± 4.2 and -19.66 ± 3.35 kJ mol^{-1}, for purified and unpurified CypA, respectively [32] were 3/2 of the ESI-SUPREX values found here. Differences of ESI-SUPREX determined $C_{1/2}$ values for apo CypA, CypA/MeOH and CypA/CsA are within a reasonable margin of the experimental error (Table 2). We can attribute the addition of methanol, different buffer conditions, and unknown recombinant differences in the protein fold (as suggested by the split peaks in our direct infusion experiments Fig. 2C) to the discrepancy of absolute ΔG_f values, and then the resulting $\Delta \Delta G_f$ values are within the error associated with the SUPREX approach. This culminates in the differences in reported dissociation constants. It is conceivable that these errors are due to the inherent experimental errors; if we take the approach shown in Ref. [32] and average the m-values obtained in all SUPREX experiments we obtain a K_d of 7.11 ± 0.29 nM (Table 2) which agrees remarkably well with previously published data. However there are caveats to this approach for our data. The decision to interpret each m-value individually is supported by differences in the absolute values of the three systems. The m-value represents the slope of the $-RT \ln((k_{int}t/0.693) - 1)$ *versus* $C_{1/2}$ plot and contains information of the surface exposure of the protein in question during the unfolding event. In Ref. [32] almost identical m-values have been reported, hence averaging there might be justifiable. By contrast, our data gives different slopes (m-values) which may be due to different conformations in each set of experimental conditions, rather than to experimental error.

3.3.4. Gas phase HDX

Following exposure of CypA to deuterated methanol in the ion trap, a graph of deuterium uptake with time (10–100 s) can be plotted as shown in Fig. 5. The data here was obtained from the ion [CypA+7H]$^{7+}$ and was subsequently 'deconvoluted' to give the mass increase for the neutral protein.

The overall deuterium uptake over the first 11 s is linear and rapid after which, the uptake slows. A reduction in solvent exposure infers the presence of a stable intermediate with limited or no further unfolding over the following 6.5 s. The conformer is destabilised after 17.5 s and another stage of rapid exchange ensues, a result of what is interpreted as an unfolding or refolding event. After 30 s the exchange starts to plateau, although there continues to be a slight increase in deuterium uptake, reaching a maximum around 180 Da, a value equivalent to the maximum solution phase exchanges in deuterated ammonium acetate buffered CypA (Table 1) where CsA has been co-incubated, but less than that seen for CypA by itself. The most likely explanation being that the protein unfolds so far in the first 30 s and then undergoes hydrophilic collapse preventing further exchange. Any relay mechanism initiating exchange at this point will be considerably hindered or even arrested.

These conclusions are supported by studies on ubiquitin and cytochrome C by Clemmer and co-workers who compared conformers of equivalent charge states by ion mobility mass spectrometry (IM-MS) and ions trapped for some number of seconds [39,40]. IM-MS determined that the collision cross-sections of each protein at different charge states were related to the degree of unfolding. In these studies ions were trapped for specified amounts of time either with a double drift cell experiment or with a Paul trap drift cell apparatus, and the ensuing conformers separated by ion mobility. A folded structure may unfold but will ultimately migrate into a gas stable conformation. As the trapping time was increased, multiple unfolded intermediates were detected until a point came when they collapsed into a compact structure. It was also ascertained that structures in both the mobility cell and trap do not reach equilibrium, i.e. once unfolded in vacuum, the process does not reverse. This point has been reinforced recently by Breuker and McLafferty [41], who with evidence from a number of studies conclude that the gas phase will ultimately cause structural alteration to the solution conformations of proteins. Here we can assume that the conformations of CypA for $z = +7$ are not significantly unfolded as they enter the trap, and this is supported by the fact that we observe a $z = +7$ CypA:CsA complex, but it is certainly possible that some unfolding and refolding/annealing of CypA occurs which is lessened in the presence of CsA.

In our solution phase work described above, deuterium uptake by CypA is reduced when CsA is present, and the protein is stabilised with respect to unfolding. This result is reiterated in the gas phase HDX experiments and supports the work of Yan-Hong et al. [35] suggesting that CsA forms a strong interaction with CypA such that exchange is considerably slowed. The natural log of hydrogen depletion against activation time in the trap was plotted to compare deuterium uptake of CypA, Cyp A released from the CypA:CsA complex and CypA in the absence of ligand (Fig. 6). This analysis was performed on undeconvoluted data. Exchange rates were calculated by linear fitting over two regions of the plot.

Isolation of the complex results in partial dissociation which releases apo CypA with charge state 7+. It is plausible that in the absence of external factors (solvent, buffer ions, etc.) this species may retain a memory of its complexed state. Maximum uptakes of deuterium for the unbound product [CypA+7H]$^{7+}$ is nearly 8 deuteriums less than for that found for [CypA+7H]$^{7+}$ originating from ligand free solution. This implies the structure either (a) collapses in on itself in the solvent free environment when the CsA is released on a time scale that is more rapid than the rate of exchange or, (b) the complex structure is more constrained when bound to the ligand and this conformation *is retained* on release of the CsA, i.e. the protein exhibits solvent memory. This effect is even more pronounced with [CypA+CsA+8H]$^{8+}$ where complex is retained during isolation and exchanges ~12 fewer deuteriums than ligand free
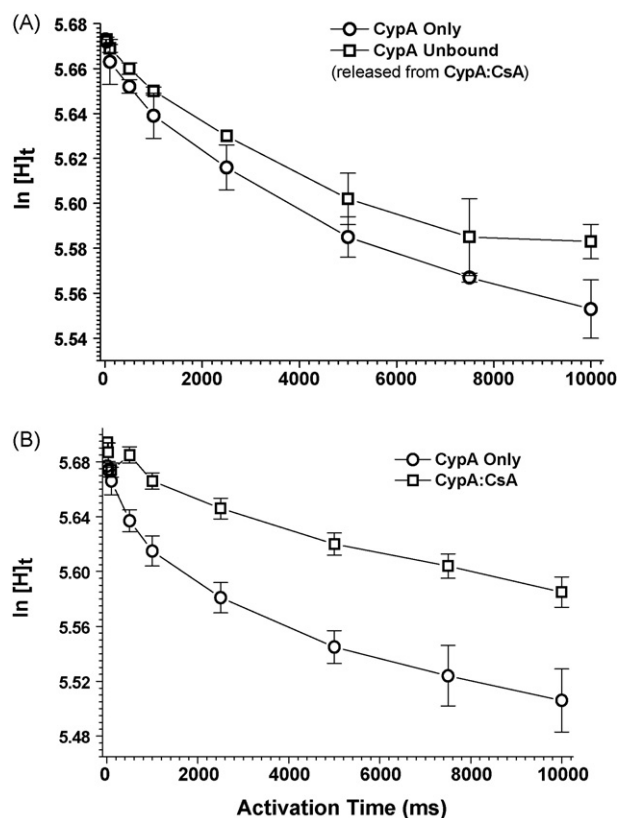
Fig. 6. Hydrogen depletion on (A) $[Cyp+7H]^{7+}$ comparing CypA only, CypA 'released' from CypA:CsA and (B) $[Cyp+8H]^{8+}$ comparing CypA only and the complex with a capillary temperature of 125 °C.

$[CypA+8H]^{8+}$. This suggests that binding CsA provides a more stable or less conformationally dynamic structure.

Retaining ions in the trap for prolonged periods of time results in a gradual increase in mass. This may be accompanied by unfolding of the structure leading to a more solvent exposed protein. Preliminary data here indicates the presence of one stable intermediate formed around 15 s. After the formation of this intermediate deuterium uptake slows dramatically for 6 s.

The maximum deuterium uptake achieved by CypA in the gas phase after 100 s was ~180 deuteriums. This is in agreement with the results obtained with deuterated ammonium acetate buffered CypA in solution and with CypA co-incubated with CsA.

4. Comparison of techniques

Each of the techniques we have employed has some merits. SUPREX and PLIMSTEX have provided K_d values that are within an order of magnitude of those reported by other techniques. However the errors with both techniques are large. Many sets of data must be taken with both approaches and data analysis is also lengthy, raising questions about the applicability of these techniques to rapidly screen proteins for ligand binding sites. For PLIMSTEX in particular the small Δm_i obtained for the CypA:CsA system, does not bode well for the use of this approach for screening of less tightly bound smaller ligands, and/or when there is any allosteric conformational change due to ligand binding. When we average out the data obtained from our ESI-SUPREX investigations, we find a K_d that is in very good agreement with that published previously for this protein–ligand system, however each individual set of ESI-SUPREX experiments have very high errors associated with them. We conclude that viable SUPREX analysis necessitates many analytical repeats, which may lessen the applicability of this approach for

high throughput screening. Although averaging the m-values under different experimental conditions provides a K_d that compares well with that reported by others, the work shown here occupied our MS platform for a total of 4 weeks running for an average of 20 h/day, to determine this single K_d.

Gas phase HDX on the protein and protein complex sprayed from buffered solution provides interesting insights into the intrinsic stability of the CypA:CsA binding site in a solvent free environment. Our findings suggest that the protein fold is preserved in the gas phase from solution for some seconds and that the protein then reorders. We also observe that CypA that has been sprayed in the presence of CsA exchanges less, even as when observed as an apo species, pointing to a conformational memory of the holo state. This approach could have more general applicability as a screen for ligand binding,

An ideal approach to understanding the differences between CypA with and without CsA bound might employ the following stratagem. A strategy of direct analysis comparing deuterium uptake by native CypA and CypA:CsA by monitoring the total mass shift. Whilst this provides a view on the role CsA plays on the stability of CypA in solution, another method such as PLIMSTEX could then be employed to provide complementary evidence from HDX of solution phase binding. Finally, having established the ability to maintain a native-like structure of CypA and its binding partners in a solvent free environment, gas phase exchange studies are also utilised to compare H/D exchange of the apo- and holo protein.

Stable intermediate conformers were not detected by any of the above techniques and we have assumed therefore that these experiments are conducted in an EX2 regime where the refolding of the protein is faster than the H/D exchange rate of unprotected amide protons. This assumption is more valid in the presence of CsA, although absence of distinct peaks during HDX does not necessarily signal that the exchange occurs in EX2 regime, rather that the exchange is uncorrelated. It is possible for the uncorrelated exchange to be observed under EX1 conditions which would be exchange due to local fluctuations, each of which exposes only a small amount of labile hydrogen atoms [42].

Kipping and Schierhorn [43] monitored CsA exchange rather than CypA by MALDI-ToF. CsA has only 4 amide hydrogens all of which exchange rapidly in solution. They were able to prove the existence of an H-bond in the Abu pocket of the CypA binding site by its ability to significantly reduce exchange to less than 11%.

Comprehensive H/D exchange studies performed on CypA by Yan-Hong et al. [35] utilised SEA HSQC (solvent exposed amide – heteronuclear single quantum coherence) and identified four exchange populations. They also showed that 80% of the residues occupying the CsA binding pocket have k_{ex} values $>1 \times 10^{-4}\,min^{-1}$, and that *all* the residues in the binding site are flexible and as such favour complex formation. Using these findings, one would expect to see the deuterium uptake of native CypA by MS to be faster than for its CypA:CsA counterpart and indeed all of the techniques reported here do show that.

5. Summary

We have taken an extremely well know protein–ligand complex and studied it with 4 different HDX approaches. Each approach has some merits, the direct infusion gives a very clear visual reference to the stabilisation of the protein by a ligand, and rates of exchange provide a global view of the conformations adopted in solution. Although it has been shown to provide data on ligand binding, we show here that for the CypA:CsA system, PLIMSTEX here gives a result with associated errors that are within the margin of the deuterium uptake due to protection upon ligand binding. The mass shift due to ligand stabilisation is rather small. These find-

ings emphasise the necessity of measurable amide protection. The larger this protection is the more reproducible and reliable the data will be. This renders PLIMSTEX highly system dependent and as such perhaps less suited to *a priori* protein–ligand high throughput screening. Automated SUPREX for the CypA–CsA complex exhibits good data reproducibility and accuracy within reported margins for SUPREX. Recombinant human CypA which comprises an additional N-terminal methionine, complexed with CsA demonstrates good agreement for ΔG_f and m with data previously reported. The pre- and post-transition baselines and the $C_{1/2}$ SUPREX values of CypA incubated in 2.3% methanol suggest an alcohol induced denaturation and stabilising effect although a weakening of the CypA:CsA interaction compared with other findings. The developed automated ESI-SUPREX methodology demonstrates the capability of K_d determination within a reasonable margin to previously reported values, and if we average all of the data we get a remarkable agreement with the K_d values of others. Many synthetic ligands must be incubated with protein in the presence of an organic solvent, our findings here with methanol cast a little doubt on the suitability of this method for general ligand screening. Nevertheless, with well investigated and well behaving enzyme–substrate systems good quality data can be achieved, albeit it at the expense of high number of analytical repeats.Finally the work using HDX in the gas phase on proteins sprayed from aqueous buffered solutions has promise as a route to gain information on their native conformation(s) This may well be of increasing importance with the rise in top-down sequencing methodologies to determine both primary and tertiary protein folds.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.05.028.

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