Analytical Techniques

Direct Characterization of Enzyme–Substrate Complexes by Using Electrosonic Spray Ionization Mass Spectrometry**

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Numerous reports have cited the use of electrospray ionization (ESI) mass spectrometry to characterize intact, multiply-charged proteins and protein complexes.[1–4] Herein we describe the application of a variant method, electrosonic spray ionization (ESSI),[5] to monitor dynamic changes associated with formation of binary and ternary complexes, such as enzyme–substrate and enzyme–substrate–inhibitor systems. The basis for the experiment is that the gentle ESSI ionization method preserves solution protein and protein complex structures and tags each protein with a charge state that is characteristic of its conformation. In the course of the study, we show that noncovalent solution interactions between the enzyme imidazole-3-glycerol phosphate synthase (IGP synthase) and the substrate/inhibitor are preserved in the gas-phase ions. This enzyme contains two distinct active sites, the glutaminase domain and the synthetase domain located some 30 Å apart. The observations indicate the capacity of this method to preserve native-like protein

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conformations, and also to detect minor alterations in the protein structure induced by ligand binding as well as major alterations associated with denaturation.

Many regulatory events in living systems are governed by protein interactions involving either static or transient noncovalent macromolecular complexes. These interactions are often mediated through subtle changes of protein structure that translate into catalytic properties or signaling events within a cell.[6] These interactions between proteins and/or small-molecule metabolites generally involve changes in surface accessibility through ligand-induced conformational changes. Effectors, such as drugs or cellular second messengers, can perturb these interactions either directly or indirectly and their potency is often related to the extent to which these protein conformations are stabilized. Often indirect kinetic measurements or spectral methods must be used to deduce information regarding the dynamics of the protein–ligand or protein–protein interactions.

Since the introduction of the "soft" ionization techniques, electrospray ionization[7] (ESI) and matrix-assisted laser desorption ionization[8] (MALDI), the study of noncovalent biological complexes has been a focus of significant attention.[1,4,9–11] Electrospray ionization, in particular, allows large macromolecular assemblies to be ionized directly from solution and offers the opportunity to analyzing these complexes. Owing to multiple charging inherent in the electrospray process, these ions have relatively low mass/charge ratios and are amenable to characterization using ion-trapping instruments. There are a number of recent examples employing ESI/MS-based methods for detecting specific noncovalently bound protein assemblies and linking their solution-phase properties to those of the detected gas-phase ion.[2,4,9,12] Recently, we introduced a new electrospray variant, electrosonic spray ionization (ESSI),[5] for ionizing proteins from aqueous solutions at physiological pH values. The technique showed considerable improvement in spectral peak widths when compared to the nano-electrospray technique. Evidence was provided that the observed narrow charge-state distributions correspond to native-like solution-phase structures.[5] This result suggests a capability of ESSI for preserving intact biomolecule complexes in solution, transferring them to the gas phase, and detecting whether the native structure is retained. Herein, we test this hypothesis in a particular case of an unusual enzyme–substrate system. Direct characterization of the binary and ternary enzyme–substrate complexes is demonstrated and the solution-phase binding order is deduced from the pattern of charge states observed.

IGP synthase (IGPS) is a bifunctional enzyme of the glutamine amidotransferase family that incorporates ammonia derived from glutamine hydrolysis into the nucleotide, *N*-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR) to yield 5′-(5-aminoimidazole-4-carboxamide) ribonucleotide (AICAR) and imidazole glycerol phosphate (IGP).[13] This glutamine amidotransferase exhibits a $(\beta/\alpha)_8$ motif with the nucleotide active site formed by loop regions at the open end or top of the barrel. Such enzymes are excellent targets for genetic redesign, since the substrate specificity and catalytic function resides in the loop regions which are separated from the α helices and β strands that provide structural stability. A second active site for glutamine binding and hydrolysis (glutaminase site) resides in a separate domain juxtaposed at the bottom of the barrel some 30 Å away from the nucleotide binding site. The efficient transfer of the ammonia generated in this second active site to the nucleotide binding site requires an orchestrated signaling event that transmits binding and catalytic information across the interface of these two protein domains. However, these events have evaded any type of direct observation.

The ESSI mass spectrum of the intact 61 kDa IGPS is shown in Figure 1 and characteristically it shows a narrow charge-state distribution with the majority of the protein's

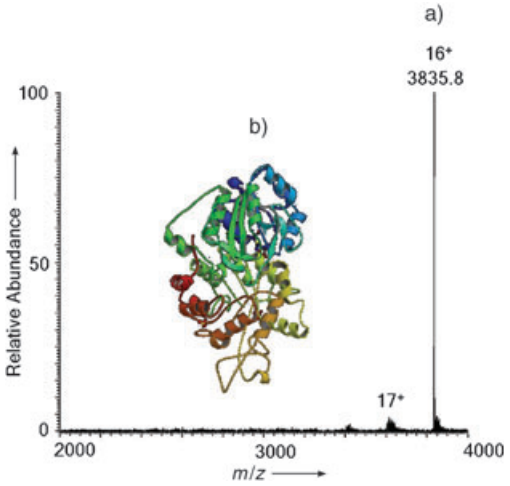

Figure 1. a) ESSI mass spectrum of imidazole-3-glycerol phosphate synthase (IGPS) in 10 mM ammonium acetate pH 7.1. b) 3D crystal structure of IGPS (http://www.rcsb.org/pdb/, Protein Data Bank Identifier (PDB ID): 1JVN[18]).

charge being collapsed into the +16 charge state. There is evidence that the observed charge state in ESI corresponds to the protein's net ionic charge in solution.[14] In addition, narrow charge-state distributions can indicate the retention of compact structures in the gas-phase that are like those of the native solution state.[11,15]

The observation of fewer charge states and, in some cases, a single charge state for a protein enhances the ability to track subtle changes occurring from perturbations of the enzyme–substrate system, as well as making it easier detect small molecular complexes.[5] Shown in Figure 2 is the ESSI mass spectrum acquired from a solution of IGPS and rPRFAR, the reduced form of PRFAR. The noncovalent complex of the enzyme and nucleotide substrate inhibitor is preserved in the gas phase and gives a single charge state, +16. In comparison, when using ESI the peaks are broader and less intense and as energy (that is, high capillary temperature and tube lens offset voltage) is added to enhance desolvation, dissociation of the complex is observed (data not shown). In Figure 2 the nucleotide inhibitor is not observed to be bound to the other higher charge states of the protein even though these appear in the mass spectrum. These charge states, +17 to +19,

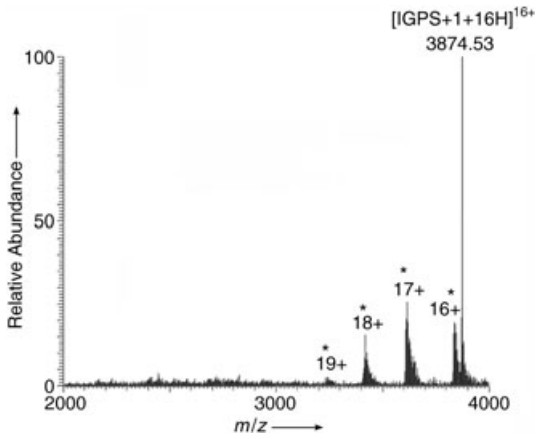

Figure 2. ESSI mass spectrum of IGPS–rPRFAR (**1**) solution containing 10 mM ammonium acetate pH 7.1 and 1 mM piperazine-*N*,*N*′-bis(2-ethane sulfonic acid) (PIPES) buffer. The (*) indicates peaks assigned to alternative conformations of the IGPS characterized by higher charge states.

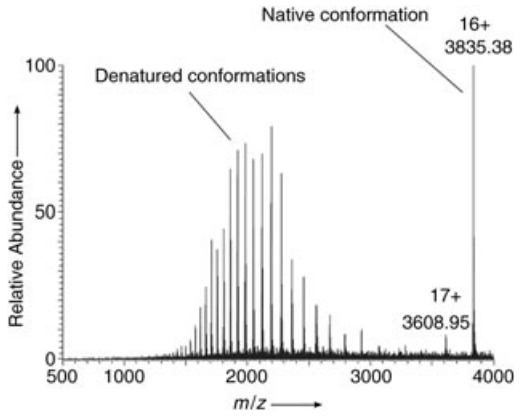

Figure 3. ESSI mass spectrum of IGPS under denaturing conditions. The presence of strong signals arising from multiple higher charge states is indicative of open denatured conformations although the single peak characteristic of the native structure is still present.

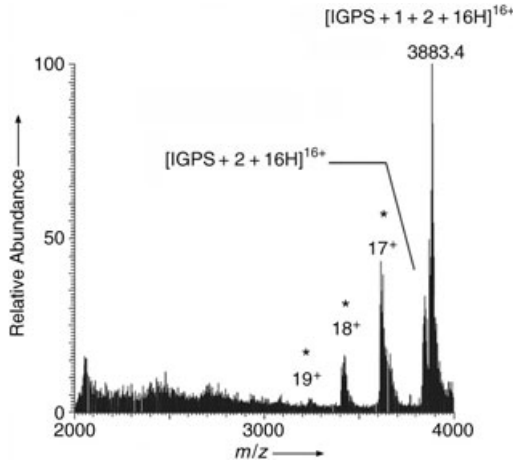

Figure 4. ESSI mass spectrum of IGPS–rPRFAR (**1**) acivicin (**2**) solution containing 10 mM ammonium acetate pH 7.1 and 1 mM PIPES buffer. The (*) indicates peaks assigned to an alternative non-native and hence nonbinding conformation(s) of the IGPS enzyme distinguished by higher charge states.

were virtually absent for the pure protein (Figure 1) and are interpreted as representing the protein in conformations that are unfavorable for substrate binding. (The unbound +16 signal marked with an asterisk is similarly assigned.) These conformations may result from binding–release events in which the released structure is altered and trapped in an unfavorable state through the course of the experiment. The following information is consistent with this interpretation. The binding of the nucleotide inhibitor induces small conformational changes within the $(\beta/\alpha)_8$ barrel of the enzyme.[13] An electrostatic gate of alternating positively and negatively charged amino acids is present in the interdomain region of the enzyme. Residues R239, E293, K360, and E465 make up this cap which facilitates ammonia tunneling within the hydrophobic core of the protein.[13] The X-ray crystal structure reveals the presence of a water-filled cavity near the interdomain region of the enzyme.[16] As substrate binding occurs in the synthase domain, a conformational reorganization of residues in the interdomain region takes place, exposing basic residues to solvent-accessible regions where they may be protonated. The increase in the number of charge states appearing in Figure 2 is tentatively associated with this conformational reorganization. This phenomenon, however, is clearly not a sign of global unfolding which gives the much broader charge-state distribution characteristic of a more open structure as is displayed in Figure 3. The observed peak broadening in Figure 2 for the asterisked charge states +16 to +19 implies the formation of a number of loose solvent or buffer adducts. Note that the spectrum shown in Figure 3 was taken from a solution containing 6 mM PIPES which is a zwitterionic, non-volatile buffer compound and presumably makes stable ion pairs which contain protonated arginine residues.

Figure 4 shows the ESSI mass spectrum of a solution containing IGPS, rPRFAR, and the glutamine amidotransferase inhibitor, acivicin. The peak at *m*/*z* 3883 corresponds to the ternary complex of IGPS, rPRFAR, and acivicin. The preservation of the products of two separate noncovalent binding events for the +16 charge state provides evidence for the existence of more and less favorable conformations in protein systems, recognized in this case by their characteristic charge states. Note that IGP synthase did not form a stable complex in the presence of acivicin alone. This is consistent with the fact that nucleotide binding induces a 200-fold shift in the apparent affinity of acivicin for IGPS.[17] As such, it is concluded that these binding events are not a result of non-specific interactions; rather, they are indicative of the interactions occurring in solution.

In conclusion, these studies reveal the feasibility of using electrosonic spray ionization (ESSI) for ionizing intact biomolecular complexes and preserving their solution-phase interactions in the gas phase. The example described herein emphasizes the advantages of ESSI in the direct characterization of proteins interacting noncovalently with multisubstrate systems. The identification of ligand binding to selected charge states highlights the potential of this ionization

method to detect alternative protein conformations in equilibrium with the native conformation at concentrations that would evade detection by other biophysical methods.

Experimental Section

The electrosonic spray ionization source has been described in detail elsewhere.[5] The high voltage was applied to the stainless steel syringe needle used for sample infusion. The source and instrument parameters used in this study are listed in Table 1. The position of the ESSI source was carefully aligned in front of the heated capillary interface of the Finnigan LCQ Classic ion trap used in this work. The atmospheric interface potentials and lens potentials in the fore vacuum were optimized for maximum ion intensity. For this study, the ion trap was operated in high-mass mode to allow mass analysis up to 4000 Th. IGPS and rPRFAR was prepared as described in reference [14] and references therein. The purified protein was stored in 50 mM PIPES buffer until required for analysis. After purification, IGPS was buffer exchanged using Microcon YM-10 (10 kDa M_r cutoff) membrane filters (Millipore) against 0.2 M ammonium acetate pH 7.1 and reconstituted in 10 mM ammonium acetate pH 7.1 to a final concentration of approximately 1 μM. The centrifugation cycles were repeated 3×. For this study rPRFAR (**1**) a competitive nucleotide inhibitor of IGPS, and acivicin (**2**) [(*RS*,5*S*)-*R*-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] (Sigma), a covalent inactivator of the glutaminase function, were used as substrate analogues for IGPS.[15] Both rPRFAR and acivicin were prepared in PIPES buffer to a concentration of 6 mM. rPRFAR concentration was determined by UV-spectroscopy using an extinction coefficient of $6069\,M^{-1}\,cm^{-1}$ at 300 nm. Mixtures of the enzyme and substrate(s) were prepared to an enzyme:substrate ratio of 1:100.

Table 1: Experimental parameters used with the LCQ Classic mass spectrometer.

Parameter	Value
Sample flow rate	$3\ \mu L\,min^{-1}$
Nebulizing gas linear velocity	$350\ m\,s^{-1}$
Spray potential	3000 V
Heated capillary temperature	150 °C
Heated capillary voltage	15 V
Tube lens voltage	110 V
Octapole 1 offset	−1.3 V
Lens potential	−25.1 V
Octapole 2 offset	−3.0 V
Automatic Gain Control Target (AGC)	5E+07

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