Mass Spectrometric Determination of Association Constants of Adenylate Kinase with Two Noncovalent Inhibitors

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Noncovalent complexes between chicken muscle adenylate kinase and two inhibitors, P^1,P^4-di(adenosine-5')tetraphosphate (Ap4A) and P^1,P^5-di(adenosine-5') pentaphosphate (Ap5A), were investigated with electrospray ionization mass spectrometry under non-denaturing conditions. The nonconvalent nature and the specificity of the complexes are demonstrated with a number of control experiments. Titration experiments allowed the association constants for inhibitor binding to be determined. Problems with concentration dependent ion yields are circumvented by a data evaluation method that is insensitive to the overall ionization efficiency. The K_a values found were 9.0×10^4 M^{-1} (Ap4A) and 4.0×10^7 M^{-1} (Ap5A), respectively, in very good agreement with available literature data.   (J Am Soc Mass Spectrom 2003, 14, 442–448) © 2003 American Society for Mass Spectrometry

Soft ionization mass spectrometry (MS), in particular matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), is well known for its ability to bring high molecular weight biomolecules into the gase phase and to even preserve noncovalent complexes [1–10]. Besides its established applications, a recurring question concerns the applicability of mass spectrometry to measuring noncovalent interaction strengths [11]. There are different approaches to measure the interaction strengths in the gas phase as well as in the liquid phase. In the former case, the mass spectra should ideally directly represent the solution phase chemistry. Gas-phase methods include cone voltage driven dissociation (called the "VC_{50}" method) [12, 13], collision-induced dissociation [14, 15], guided ion beam tandem mass spectrometry [16, 17], blackbody infrared radiative dissociation [18, 19] and heated capillary dissocation [20, 21].

Some mass spectrometric studies have been reported in the literature for the determination of noncovalent interaction strengths. There are three basic methods to determine association constants that are reflective of those in solution: first, it is possible to record "melting curves" by raising the temperature of the analyte solution and to use MS to determine the percentage of intact complexes. Cheng et al. analyzed complementary DNA strands [22] and Fändrich et al. studied the chaperone GimC/prefo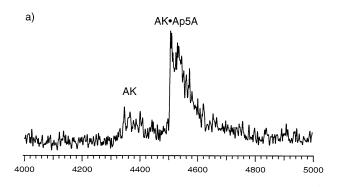lding homologue complex [13] with this method. Second, competition experiments can be used to evaluate the stability of noncovalent complexes. In Jørgensen's method [23, 24], the relative intensities of the free host and the different complexes were used, whereas Kempen et al. employed the known concentration of a reference complex to determine the concentration of an unknown complex [25]. Third, a titration of a host with a guest (or vice versa) can be used. In this case, the mass spectrometric signals should accurately represent the concentrations of the species in solution. The titration method is documented in a number of publications, all of them convincingly showing the successful use of mass spectrometry for the quantitative determination of noncovalent binding constants of proteins and small molecules [26–33]. This aspect was the main focus in most previous research; the specificity of the noncovalent interaction was often not verified. However, it is essential to establish that the analyzed complexes are neither gas phase clusters nor the result of unspecific adduct formation in solution. Another, equally important aspect concerns the ionization efficiency of the species appearing in the mass spectra. If a calibration curve is first recorded for one species to evaluate its concentration, the total ionization efficiency must remain the same during a subsequent titration experiment. For some systems this may be true, but often, the total ionization efficiency drops as the titration proceeds. Thus, a measurement that is independent of total ionization efficiency would be preferable.

In an effort to develop soft ionization mass spectrometry into a more accepted and robust method for studying noncovalent interactions quantitatively, we present a method where only the relative signals of a

protein and a noncovalent protein-inhibitor complex are determined. In other words, a varying overall ionization efficiency will not affect the results. Furthermore, we present a set of control experiments to verify the specificity of the complex and to rule out any unspecific or gas phase adducts. The system studied in this work was adenylate kinase (AK) from chicken muscle and its two noncovalent inhibitors P^1,P^4-di(adenosine-5')tetraphosphate (Ap4A) and P^1,P^5-di(adenosine-5')pentaphosphate (Ap5A). AK is a phosphotransferase that catalyzes the transfer of the γ-phosphate group of magnesium-adenosine-triphosphate (MgATP) to adenosine-monophosphate (AMP) and therefore plays an important role in the energy metabolism of cells [34]. There are five major types of AK: AK1 (from cytosols of mammalian or vertebrate muscles, with c, h, p, and r designating chicken, human, porcine, and rabbit, respectively), AKe (from *Escherichia coli*), AKy (from yeast), AK2 (from mammalian mitochondrial intermembrane space), and AK3 (from mammalian mitochondrial matrix, specific to GTP instead of ATP). AK1 differs from AKe and AKy in lacking a 30-residue loop near position 132 [35]. In all other aspects of structure and function, AK1 and AKe show high similarities [36]. AK has a molecular weight of 21.6 kDa and its inhibitors are well studied. It is thus a well-suited model system for the studies presented here.

Experimental Section

Instrumentation   All experiments were conducted on a prototype electrospray orthogonal injection time-of-flight mass spectrometer from Agilent (Palo Alto, California, USA). It consists of a commercial atmospheric pressure electrospray interface (Agilent), two skimmers, and an octapol ion guide for collisional cooling. The ions are extracted orthogonally by a pulsed extraction field at a repetition rate of 4 kHz. The ions are guided via a single stage reflectron to the detector (ETP Electron Multipliers Pty Ltd., Ermington, Australia). The detector signal is amplified and digitized with a computer (Hewlett Packard, Palo Alto, California, USA) at a sampling frequency of 250 MHz. Infusion rates of 3 to 5 μl/min with a pneumatically assisted ESI source ("ion spray") were used. The spray potentials were between 2700 and 3000 V. The source conditions of the instrument were optimized for the transfer of high molecular weight ions and for gentle desolvation conditions by adjusting the different source and transfer potentials. For low energy dissociation experiments the voltage between sampling cone and skimmer was increased, from 32 V to 140 V, which is sufficient to dissociate noncovalent complexes. Spectra were recorded with a repetition rate of 4kHz and a couple of 100'000 spectra were summed and transferred to a PC for further processing.
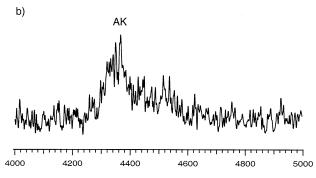

Figure 1. ESI mass spectra (charge state +5) of 10μM AK and 10μM Ap5A in 50mM (HNEt$_3$)HCO$_3$ with **a**) soft ionization conditions and **b**) harsher ionization conditions (increased cone voltage) leading to low energy dissociation.

Materials and Methods

Adenylate kinase (M = 21689 Da) from chicken muscle was obtained from Sigma (St. Louis, MA, USA) and used for the titration experiments without purification. However, even in "essentially salt free" AK samples, a batch dependent amount of magnesium was present. The sample were thus treated by adding EDTA, primarily to allow comparison of our results with literature data. The solvent for all samples was water. NH$_4$AcO and (HNEt$_3$)HCO$_3$ were used to buffer the protein-inhibitor solutions; these are well known for conserving noncovalent interactions in ESI [37]. The titration samples contained between 8.8 and 9.0 μM AK (depending on the volume of added inhibitor), 0 to 9.6 μM P^1,P^5-di(adenosine-5')pentaphosphate (Ap5A) or 0 to 12 μM P^1,P^4-di(adenosine-5')tetraphosphate (Ap4A), 50mM (HNEt$_3$)HCO$_3$, and 500 μM EDTA. Inhibitors were from Fluka (Buchs, Switzerland) and from Sigma (St. Louis, MO/USA), respectively. 8-N$_3$-ATP was obtained from Biolog Life Science Institute, Bremen, Germany.

AK was denatured in four different ways. A solution containing 10 μM AK in 10mM NH$_4$AcO buffer was incubated with 50% MeOH (HPLC grade) or 5% AcOH (reagent grade), prior as well as after adding the 10μM Ap5A. For the low energy dissociation experiments described in Figure 1 (see below), several different solutions were used, including 107μM AK/10μM Ap5A in 50mM (HNEt$_3$)HCO$_3$ buffer and 10μM AK/20μM Ap5A in 5mM NH$_4$AcO buffer.

AK was covalently modified in its binding pocket according to a slightly adapted method originally published by Olcott [38] and David [39]. 0.3 μl 5mM 8-N_3-ATP in 10mM NH_4AcO, 0.5μl 20mM $MgCl_2$, 12μl 250μM AK in 10mM NH_4AcO and 87 μl 50mM Tris/HCl (pH = 7.2) were mixed on ice and incubated for 30 seconds. The solution was exposed to UV radiation at a wavelength of 254 nm (Westinghouse Electric Corp. Fluorescent & Vapor Lamp Division, Broomfield, N.J., USA) for 75 seconds. Another 0.3 μl 5mM 8-N_3-ATP in 10mM NH_4AcO was added and again exposed to the UV radiation for 75 seconds. The Tris/HCl buffer was exchanged with a NAP-5 gel permeation column (Pharmacia Biotec AB, Sweden) to 10mM NH_4AcO. The reaction was incomplete, but the activity of the treated AK sample was probed by an essay described by Wallimann et al. [40] and was found to be reduced by 25% $\pm$ 5%. The mass of the modified AK was 22259 Da.

Data Evaluation   Equations 1 through 3 describe the complex formation of AK with its inhibitor ApXA:

$$K_a = \frac{[AK \cdot ApXA]}{[AK] \times [ApXA]} \tag{1}$$

$$[AK]_0 = [AK] + [AK \cdot ApXA] \tag{2}$$

$$[ApXA]_0 = [ApXA] + [AK \cdot ApXA] \tag{3}$$

where K_a is the association constant, [AK] the concentration of the bare protein, [ApXA] the concentration of the inhibitor and [AK·ApXA] the concentration of the complex. "0" denotes the total concentration of a given species. Experimentally, we measure the concentration ratio of the complex and the bare protein, which is called R below:

$$R = \frac{[AK \cdot ApXA]}{[AK]} \tag{4}$$

These equations can be solved for K_a for known values of $[AK]_0$ and $[ApXA]_0$ to yield eq 5:

$$K_a = \frac{R^2 + R}{[ApXA]_0 \times (1 + R) - [AK]_0 \times R} \tag{5}$$

A more robust, global data evaluation that takes into account all data points can be achieved by a least squares fit of the experimental R vs. known $[ApXA]_0$ values to an expression which is obtained by solving the above equations for R. This yields eq 6, where K_a is the only adjustable parameter.

$$R = \frac{1}{2}\left(-1 - K_a \times [AK]_0 + K_a \times [ApXA]_0 + \sqrt{4 \times K_a \times [ApXA]_0 + (K_a \times [ApXA]_0 - K_a \times [AK]_0 - 1)^2} \right)$$

$$\tag{6}$$

Particularly for statistical error analysis, the fit described in eq 6 is preferable to a Scatchard plot (see, for example [32]) or other forms of data linearization that are used for graphical evaluation of protein inhibition. A similar approach as the one described here was chosen by Greig et al. [26] who used a fit to a second order polynomial to determine two dissociaton constants of a BSA–oligonulecotide complex.

In the approach described here, the ratio R of peak areas for the [AK] and [AK·ApXA] signals is used to determine the association constant. A good baseline correction method is important to obtain reliable resluts, in particular when one of the peaks is hardly visible. Different baseline correction methods were therefore tested. In the simplest approach, a constant value was subtracted from the data. In a more refined method, a line was fitted to the baseline allowing the correction of a sloping baseline. In the third method, a linear baseline was calculated for each peak, allowing for different slopes in the baseline. In the most sophisticated method, an arbitrary mathematical form of the baseline was allowed. The differences between these methods were found to be negligible. Therefore the simplest baseline correction method was used throughout.

Each titration point was measured five times. The +5 charge state of the bare protein and the complex were integrated. Other detectable charge states had negligible signal intensities. The ratio R was calculated for each spectrum. A least square fit was performed using equation 5 with K_a as the fitting parameter for the titration experiment.

Results and Discussion

For analysis of large noncovalent complexes, suitable conditions must be found where desolvation is sufficiently complete to allow mass resolution of all different species, but where the noncovalent complex is not destroyed. This compromise is responsible for the fairly low S/N ratio, which was found to drop even further with increasing inhibitor concentration. Figure 2 shows mass spectra of 8.8 μM AK with 8 μM Ap4A (Trace a) and Ap5A (Trace b), respectively. The observed peaks are all quite broad, probably due to an incomplete desolvation leading to water and buffer adducts. However, the signals of free AK and of the complexes are clearly resolved. Dependent on the sample batch, a second, less abundant peak for AK and for AK·ApXA was detected in some of the spectra. It could not be
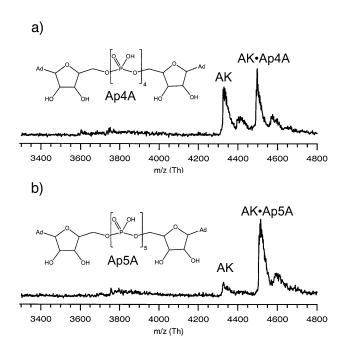
Figure 2. Spectrum of 8.8μM AK (+5 charge state) with **a)** 8μM Ap4A and **b)** 8μM Ap5A in 50mM (HNEt$_3$)HCO$_3$. The ionization and the desolvation are very soft, leading to broad peaks. Many salt and buffer adducts are observed. In the insets Ap4A respectively Ap5A is shown, whereas Ad denotes for adenine.

unambiguously identified, but we assume it to be due to a contamination from a sequence variant of AK. Since these peaks originate from an AK variant of comparable activity, we included them in the evaluation of the titration experiments as well.

If a signal at the mass of a complex is detected by mass spectrometry, it does not automatically imply that the complex is indeed noncovalent and specific. Therefore we performed a number of control experiments to

prove the specificity and the noncovalent nature of the suggested interaction. Figure 1 shows spectra of AK with the inhibitor Ap5A at physiological pH. Trace (a) was recorded with soft ionization and desolvation conditions preserving the noncovalent complex, whereas trace (b) represents harsher conditions (increased cone - skimmer voltage) leading to low energy dissociation of the complex. This data shows the +5 charge state and was recorded using 10 μM AK and 10 μM Ap5A in 50 mM (HNEt$_3$)HCO$_3$. Identical behavior was found for other solution conditions, e.g., 10 μM AK, 20 μM Ap5A in 5 mM NH$_4$AcO. The complex always disappeared under harsher interface conditions, while the protein itself remained intact. We can thus safely assume that this complex is of noncovalent nature. Further control experiments included methanol or acetic acid induced denaturation of AK both prior to and after adding Ap5A to the solution. An intact tertiary structure of the protein is needed to form and maintain the noncovalent complex. Upon denaturation, no complexes could be observed (data not shown), giving additional strong evidence for its specificity.

Finally, it should be confirmed that the inhibitor binds at the active site of the protein. Therefore, AK was covalently modified as outlined in the experimental section. In brief, the active site is blocked by covalently bound ATP mimic. The resulting sample contained both modified and unmodified AK which could be differenciated due to their different mass. Figure 3 shows the mass spectrum of this modified AK sample in the presence of Ap5A at physiological pH. Almost no inhibitor binding of the modified AK is observed, suggesting that the inhibitor Ap5A binds in the active site of the unmodified protein only. The results of all control experiments are fully consistent with specific noncovalent inhibitor binding in the active site of the
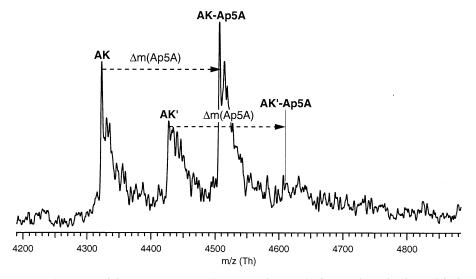

Figure 3. Spectrum of the reaction mixture (10 μM total protein) of AK and covalently modified AK (AK′) with 10μM of Ap5A (with respect to the total concentration of AK and AK′) in 50mM (HNEt$_3$)HCO$_3$. Almost no binding to AK′ is observed, suggesting that the inhibitor binds in the binding pocket of the protein.
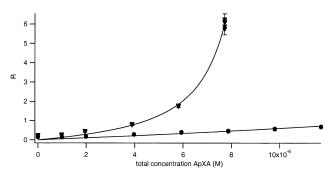
Figure 4. Result of the titration experiments of AK with Ap5A (filled inverted triangle, Ka = $4.0 \times 10^7 M^{-1}$) and AK with Ap4A (filled circle, Ka = $9.0 \times 10^4 M^{-1}$). The lines are the fitted data with eq 6.

protein. The second inhibitor, Ap4A, is much less well studied than Ap5A. For example, no crystal structure data exist for AK—Ap4A complexes. For the present investigation, we assume that Ap4A also froms a similar complex with AK, as suggested by other studies [41, 42].

For the determination of solution phase binding constants by mass spectrometry, it is necessary that the experiment satisfies two conditions. First, the ionization efficiency of the protein and the complex should be equal. Second, the noncovalent complex must survive the ionization process, the transfer into the high vacuum, and the acceleration in the TOF instrument. The first condition is probably fulfilled in the present case: identical charge distributions were found for the bare protein and the protein-inhibitor complexes. Also, the tertiary structure and the mass of the bare protein and the complex are similar. During the titration experiments, we found the total ionization efficiency to decrease with increasing concentration of the inhibitor. Since we only determine the ratio of the complex and the bare protein, this has no effect on the titration results as long as the assumption of equal ionization efficiencies for both species holds. The second condition should be satisfied, too. For this particular case, electrostatic and dipolar interactions are involved in the stabilization of the complex [43,44] forces that are expected to increase upon solvent removal (unlike hydrophobic interactions that will decrease or even vanish in the gas phase).

The association constants for the AK·ApXA system were determined as described in the "Data Evaluation" section. The experimentally obtained R-values are plotted against the total inhibitor concentrations used for the titration. In Figure 4, this plot is shown for both noncovalent inhibitors. The lines represent a fit for the association constant obtained with help of eq 6. The association constant for AK and Ap5A is $K_a = 4.0 \times 10^7$ M^{-1}, for AK and Ap4A $K_a = 9.0 \times 10^4 M^{-1}$. Because the K_a value for this particular system depends on the magnesium concentration of the solution, any remaining magnesium was masked by adding an excess of EDTA. The relative standard deviations for the titration points were between 1.6% and 5.0%. The confidence interval for K_a is difficult to estimate, but it will most probably be less than one order of magnitude. In Table 1, the values obtained experimentally are compared to data published in the literature. Values determined by mass spectrometry for AK from chicken muscle lie in the same range as literature values for different species and are therefore in very good agreement. These results also validate our control experiments and the conditions for the titration. It shows that the selected control experiments are useful and that the postulated conditions are justifiable.

Conclusions

We have shown the successful determination of solution phase association constants of noncovalent protein-inhibitor complexes by ESI mass spectrometry. Mass spectrometry has great potential for becoming a widely used method in this area due to its sensitivity and speed. This method allows screening of drug libraries with respect to their association constants to a target protein. In this case, control experiments to confirm the noncovalent nature and the specificity of the complexes are only necessary to prevent overestimations of association constants due to unspecific aggregation or covalent binding. For "misses," such controls will usually not be necessary. Furthermore, the present analysis assumes that all complexes formed in solution survive in the mass spectrometer for the timescale of the measurement, and that the ionization efficiency of the bare target protein and of the protein-inhibitor complex are equal. These assumptions are true for the present sys-

Table 1. Comparison of the association constants measured and published in the literature

Complex	Species	K_a [M^{-1}]	Reference
AK1-Ap4A	Chicken muscle	9.0×10^4	This work
AKe-MgAp4A	*Escherichia coli*	2.3×10^4	Reinstein et al. [34]
AKe-Ap4A	*Escherichia coli*	7.7×10^4	Reinstein et al. [34]
AK1-MgAp4A	Pig muscle	9.1×10^4	Feldhaus et al. [42]
AK1-Ap5A	Chicken muscle	4.0×10^7	This work
AKe-MgAp5A	*Escherichia coli*	6.7×10^7	Reinstein et al. [34]
AKe-AP5A	*Escherichia coli*	1.0×10^7	Reinstein et al. [34]
AK1-MgAp5A	Rabbit muscle	$2.8–5.0 \times 10^8$	Lienhard et al. [45]
AK1-MgAp5A	Pig muscle	3.4×10^7	Feldhaus et al. [42]

tem, but must be validated when investigating unknown systems.

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