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Utilization of real-time electrospray ionization mass spectrometry to gain further insight into the course of nucleotide degradation by intestinal alkaline phosphatase

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RATIONALE: Related with its ability to degrade nucleotides, intestinal alkaline phosphatase (iAP) is an important participant in intestinal pH regulation and inflammatory processes. However, its activity has been investigated mainly by using artificial non-nucleotide substrates to enable the utilization of conventional colorimetric methods. To capture the degradation of the physiological nucleotide substrate of the enzyme along with arising intermediates and the final product, the enzymatic assay was adapted to mass spectrometric detection. Therewith, the drawbacks associated with colorimetric methods could be overcome.
METHODS: Enzymatic activity was comparatively investigated with a conventional colorimetric malachite green method and a single quadrupole mass spectrometer with an electrospray ionization source using the physiological nucleotide substrates ATP, ADP or AMP and three different pH-values in either methodological approach. By this means the enzymatic activity was assessed on the one hand by detecting the phosphate release spectrometrically at defined time points of enzymatic reaction or on the other by continuous monitoring with mass spectrometric detection.
RESULTS: Adaption of the enzymatic assay to mass spectrometric detection disclosed the entire course of all reaction components – substrate, intermediates and product – resulting from the degradation of substrate, thereby pointing out a stepwise removal of phosphate groups. By calculating enzymatic substrate conversion rates a distinctively slower degradation of AMP compared to ADP or ATP was revealed together with the finding of a substrate competition between ATP and ADP at alkaline pH.
CONCLUSIONS: The comparison of colorimetric and mass spectrometric methods to elucidate enzyme kinetics and specificity clearly underlines the advantages of mass spectrometric detection for the investigation of complex multi-component enzymatic assays. The entire course of enzymatic substrate degradation was revealed with different nucleotide substrates, thus allowing a specific monitoring of intestinal alkaline phosphatase activity.

The sphere of the enzymatic activity of intestinal alkaline phosphatase (iAP) is already known to be multifaceted, ranging from adjustment of intestinal pH-value to downregulation of lipid intestinal absorption[1] and to effects on immunity and inflammation.[2] The impact of iAP on immunity includes its capability to dephosphorylate lipopolysaccharide (LPS), the regulation of intestinal bacterial entry into the body and its involvement in the formation of adenosine by the breakdown of adenosine-5′-triphosphate (ATP), either possessing regulatory effects on inflammatory processes.[2–4]

Besides the modulatory function of iAP in inflammation, the enzyme takes part in intestinal pH regulation, whereby a drop in pH-value leads to a reduction in the activity of the enzyme and with that to an accumulation of ATP in the intestine, in this manner provoking bicarbonate secretion by the stimulation of ATP receptors on enterocytes. The consequent rise in pH coincidently increases the activity of iAP, thus inducing an enhanced ATP breakdown followed by a reduced bicarbonate secretion.[1]

The investigation of enzymatic substrate degradation is usually conducted by means of applying colorimetric methods, which are often well established and therefore easy to handle. However, they predominantly provide information solely about either substrate degradation or product generation, resulting in a picture lacking important information or that is ambiguous.

In this regard, mass spectrometry (MS) has already proved its advantageous features, attested by the presence of a variety of studies investigating a considerable amount of different enzymes. Studies range from the determination of the kinetics of enzymes, including the assessment of inhibitor constants,[5–7] to the elucidation of noncovalent complexes[8,9] and conformational fluctuations associated with enzymatic catalysis.[10] Besides, elaborate setups like online coupled continuous flow approaches coupled with mass spectrometric detection have been applied for the finding of regulatory molecules in complex mixtures affecting enzymatic activity.[11,12]

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Concomitantly, MS detection provides the possibility to entirely capture the substrate and product trace continuously in real-time.[13] Moreover, detailed information about multiple reaction intermediates may be unveiled, which are usually not measureable with colorimetric methods.[14]

In fact the degradation of ATP by iAP has been shown to include the formation of intermediates, but in an elaborate and time-consuming procedure only monitoring single time points of enzymatic reaction and also disregarding the generation of adenosine.[15]

Because of its pH dependence, the activity of iAP has already been investigated in a wide range of pH-values using mainly the artificial substrate *p*-nitrophenyl phosphate.[16,17] However, it has been shown that the use of chromogenic substrates may significantly affect enzymatic specificity.[18] Due to the variety of important functions associated with the ability of iAP to degrade nucleotides and in view of the possibility provided by MS detection to employ a non-chromogenic substrate, enzymatic activity was tested with physiological substrates. An MS-compatible enzymatic assay was developed to enable the specific monitoring of all reaction intermediates present in the course of the reaction and to gain further insight into the enzymatic degradation of several substrates.

EXPERIMENTAL

Reagents and chemicals

Ammonium heptamolybdate (#RDHA31402) was purchased from Riedel de Häen (Seelze, Germany); malachite green oxalate (#3076) was purchased from VWR BDH prolabo (Darmstadt, Germany); poly(vinyl alcohol) (#363138), ammonium acetate (#A7330), ATP (#A2383), ADP (#A2754), AMP (#A1752), intestinal alkaline phosphatase from bovine intestinal mucosa (Enzyme Commission (EC) number 3.1.3.1., molecular weight (MW) ~160kDa) (#P7640), water LC-MS CHROMASOLV(R) (39253-1L-R) were purchased from Sigma-Aldrich (Steinheim, Germany).

Instrumentation

Photometric measurements were performed with a SLT Spectra plate reader (SLT Instruments, Crailsheim, Germany).

Mass spectrometric measurements were conducted with a MSQ Plus single quadrupole mass spectrometer (Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer, Berlin, Germany) equipped with an ESI source in the positive ionization mode. Mass spectrometric needle voltage was set at 3.5 kV, cone voltage at 75 V and temperature at 225°C. All samples were detected with a mass range of 100 to 1000 *m/z*.

Sample preparation for colorimetric determination of enzymatic activity

Reagents for malachite green assay, adapted from Henkel *et al.*[19] were always prepared freshly by thoroughly mixing a proportion of 16.7% of a 5.72% (w/v) ammonium heptamolybdate solution solved in 6 M HCl, 16.7% of a 2.32% (w/v) poly(vinyl alcohol) solution dissolved in water (Milli-Q), 33.3% of a 0.081% (w/v) malachite green oxalate solution dissolved in water (Milli-Q) and 33.3% of water (Milli-Q).

Activity of iAP was investigated by means of colorimetric detection of inorganic (free) phosphate. Due to the reaction between malachite green molybdate and inorganic phosphate under acidic conditions a malachite green phosphomolybdate complex is formed that can be directly related to the phosphate released by the enzyme's activity.

The assay was conducted in 10 mM ammonium acetate solution (pH 6.0, 7.4 or 9.0) with either ATP, ADP or AMP employed as enzymatic substrates. Ammonium acetate was selected according to the work of Hogenboom *et al.*[20] and Dennhart and Letzel,[21] who have already been able to demonstrate its applicability for the investigation of enzymatic activity.

For each experiment phosphate release was determined at seven time points within 90 min. Therefore, seven individual enzymatic assays were prepared in 10 mM ammonium acetate solution with the respective pH-values with 0.2U/mL (≙ 44.64nM) iAP and 40 µM of the respective substrate (500 µL total volume). The reaction was started simultaneously by the addition of the enzyme to all assays.

In the case of time point 0, the enzyme was added to the assay, whereupon an aliquot was removed immediately and the enzymatic reaction was terminated by addition of malachite green reagent.

In case of time points 15, 30, 45, 60, 75 and 90 min an aliquot of one preliminarily prepared enzymatic assay was withdrawn and mixed with malachite green reagent to terminate the enzymatic reaction. The remaining assay was discarded. The aliquot/malachite green mixture was incubated for 20 min at room temperature and absorption was measured at 620 nm to detect the released phosphate. All experiments were performed six to nine times on two different days at room temperature.

Data evaluation in colorimetric experiments

Controls measured with the respective substrate in 10 mM ammonium acetate solution pH 6.0, 7.4 or 9.0 without enzyme revealed no increase in absorption. Therefore, an auto-dephosphorylation of the substrates could be excluded, whereupon a blank value correction was not implemented.

Data interpretation was conducted by comparing the slopes of trend lines within the initial linear range of phosphate release. The time interval for data evaluation was minutes 0 to 15 for enzymatic assays in pH 9.0, due to the faster reaction and minutes 0 to 30 for enzymatic assays in pH 6.0 or 7.4 (cf. Supplementary Table S1, Supporting Information).

Sample preparation for MS determination of enzymatic activity

Initially, different substrate and enzyme concentrations were tested with ATP substrate at pH 7.4. The most suitable combination was identified as that which resulted in a complete degradation of the substrate within 30 min and therewith in a considerable flattening of ATP substrate trace. iAP assays were performed in positive ionization mode, to maintain comparability to other enzymatic assays already established and in this context to prospectively conduct experiments with

multiplex enzymatic assays.[22] Even though higher intensities may be anticipated in negative mode, initial intensities of the nucleotides substrates between at least 150 000 counts for ATP substrate in pH 9.0 and up to 400 000 counts for AMP in pH 7.4 were obtained in positive mode.

Due to the pH-values applied and the disparity of available phosphate groups of the substrate, intermediates and of the product, different charge states of the assay components might be present. Therefore, the negatively charged groups might lead to a signal intensity underestimation of ATP and ADP towards AMP or adenosine. Nevertheless, the intensities of all assay components were exceedingly sufficient, thus enabling the assessment of the substrate degradation by means of an exponential trend line approach, also taking into account that quantification was not intended. The activity of iAPs was examined towards ATP, ADP or AMP in 10 mM ammonium acetate solution at pH 6.0, 7.4 or 9.0, respectively. The pH-value of the 10 mM ammonium actetate solution was checked daily and was readjusted to the respective value if necessary. All experiments were conducted 5 to 11 times on two different days at 21°C.

Initially, 40 µM of the respective substrate was mixed with 10 mM ammonium acetate solution pH 6.0, 7.4 or 9.0. The enzyme concentration of assays conducted at pH 6.0 or 7.4 was set to 0.2 U/mL (≙ 44.64 nM). Due to a distinct higher enzymatic activity at pH 9.0 (see Results, Figs. 4(a) and 4(b)), the concentration of enzyme was quartered to 0.05 U/mL (≙ 11.16 nM) to retain the viability of the data-evaluation procedure via the application of an exponential trend line. Controls were measured with all possible substrate-pH assay combinations but without enzyme to be able to exclude phosphate release related to mass spectrometric settings.

Reactions were always started together with mass spectrometric recording by pipetting the enzyme into the substrate/ammonium acetate solution, followed by a thorough and brief mixing. The assay was then drawn up into a 500 µL glass syringe (Hamilton-Bonaduz, Switzerland), clamped into a syringe pump (model 11 Plus, Harvard Apparatus, Hugo Sachs Elektronik, Hugstetten, Germany) and continuously pumped with 10 µL/min through a Peek tubing (1/16" × i.d. 0.13 mm, length 410 mm) into the source of the mass spectrometer. This procedure caused a time delay until the first signal could be detected and therefore the first 3 min of MS measurements were not considered for further data evaluation. Enzymatic reaction was recorded for 30 min.

Data evaluation in MS experiments

Data were processed using Xcalibur software 2.1.0.1139 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extracted ion chromatograms (EICs) of each assay component, obtained by using a *m/z* range of ±0.5 Da to the respective calculated *m/z*-value, were summed for the following compounds: Adenosine with *m/z* 268.1 and 290.1, i.e. $[Adenosine+H]^+$ and $[Adenosine+Na]^+$; AMP with *m/z* 348.1, 370.1, 386.0, 392.0 and 408.0; ADP with *m/z* 428.0, 450.0, 466.0, 472.0 and 488.0; ATP with 508.0, 530.0, 546.0, 552.0 and 568.0, i.e. $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M–H+2Na]^+$ and $[M–H+Na+K]^+$. The EICs were smoothed with a Gaussian function using a 15-points function width. Further processing was conducted with Microsoft Office Excel 2007.

Table 1. Overview of iAP enzymatic activity towards ATP, ADP or AMP substrates at pH 6.0, 7.4 or 9.0 presented either as slopes of trend lines [Δabsorption/Δtime] for colorimetric experiments or substrate degradation rates [relative substrate degradation/min] for mass spectrometric assays along with the respective standard deviations (± value)

	Colorimetric assay			Mass spectrometric assay		
	Slope of linear trend line [Δabsorption/Δtime]			Degradation rate [relative substrate degradation/min]		
	pH 6.0	pH 7.4	pH 9.0	pH 6.0	pH 7.4	pH 9.0
ATP	$1.350 \times 10^{-3} \pm 0.164 \times 10^{-3}$	$5.856 \times 10^{-3} \pm 0.949 \times 10^{-3}$	$19.73 \times 10^{-3} \pm 2.976 \times 10^{-3}$	10.17 ± 0.734	38.10 ± 13.63	575.6 ± 58.12
ADP	$1.650 \times 10^{-3} \pm 0.197 \times 10^{-3}$	$5.033 \times 10^{-3} \pm 1.476 \times 10^{-3}$	$12.27 \times 10^{-3} \pm 1.133 \times 10^{-3}$	8.430 ± 4.379	27.42 ± 15.47	751.7 ± 128.0
AMP	$1.083 \times 10^{-3} \pm 0.075 \times 10^{-3}$	$2.467 \times 10^{-3} \pm 0.711 \times 10^{-3}$	$7.133 \times 10^{-3} \pm 0.561 \times 10^{-3}$	7.001 ± 1.175	11.48 ± 5.379	121.9 ± 32.33

At first each individual assay was normalized to 1 whereupon an exponential trend line was applied to the respective substrate trace from minute 3 to 30 for assays at pH 7.4 and 6.0 and minute 3 to 15 for assays at pH 9.0 (Table 1) resulting in Eqn. (1). The substrate degradation was observed until a plateau at a remaining intensity of 0.05 was reached, which reflected the termination of the reaction. $y = 0.05$ was therefore set as the end point of the reaction. By using Eqn. (1), the time (x) required for the enzyme to completely degrade the substrate ($y = 0.05$) was calculated, which was then inserted into Eqn. (2) to determine the enzymatic conversion rate.

$$y = a^* \ \exp(bx) \quad (1)$$

$$\text{conversion rate}\left[\text{min}^{-1}\right] = \left(c[\text{substrate}]/c[\text{enzyme}]\right)/x \quad (2)$$

Statistics

Statistical analysis was performed in the same way for photometric and mass spectrometric data sets by applying Welch's t-test, which takes into account the extent of the respective standard deviations. Assays conducted with the same substrate at different pH or assays with ATP, ADP or AMP at the same pH-value were compared pairwise. Statistical significance was assumed for *p*-values of 0.05 to 0.01 (*), very significant for *p*-values of 0.01 to 0.001 (**) and extremely significant for *p*-values <0.001 (***).

RESULTS AND DISCUSSION

Intestinal pH regulatory mechanisms and the enzyme's contribution to inflammatory processes along with a variety of other functions are associated with the ability of iAP to dephosphorylate nucleotides.

Since ATP is naturally degraded to adenosine by the stepwise removal of its three phosphate groups,[15] possible intermediate products ADP and AMP were also used as substrates (Fig. 1).

Moreover, enzymatic activity was examined at three different pH-values (pH 6.0, 7.4 and 9.0). pH 9.0 was chosen, since the enzyme possesses its highest activity in alkaline medium.[17] Besides, the alkaline pH was selected to prospectively investigate regulatory compounds affecting enzymatic activity, which was similarly done previously by Vovk *et al.*, who studied inhibition of iAP at pH 9.0.[23] In contrast, acidic pH 6.0 was selected due to pH-values present in the small intestine, that range from 5.5 to 7.5, and might even locally decrease further during intestinal inflammation.[24] In addition, pH 7.4 was chosen to represent the standard pH of physiological relevance.

Besides the employment of three different nucleotide substrates as well as three different pH-values, the activity of iAP was determined using on the one hand mass spectrometric detection and on the other a well-established photometric method. The absorption values obtained hereby are attributed to the overall release of phosphate. Besides its advantageous features like being well established and easy to handle, a main disadvantage of this procedure is the indistinguishability of the actual origin of released phosphate. A mass spectrometric assay was therefore established to comprehensively examine the course of the reaction, to obtain insight into the generation of all intermediates and to detect the final product adenosine. The gain in information provided by mass spectrometric measurements may thereby help to further elucidate pH-dependent substrate degradation and substrate preferences of iAP in a physiological context.

Colorimetric determination of iAP activity

The photometrical determination of enzymatic activity was conducted by observing the phosphate released from either ATP, ADP or AMP substrate at pH 6.0, 7.4 or 9.0.[25]

By comparing the initial increases of absorption of all assay compositions (data not shown) the most obvious differences in slopes between the substrates used were measured at pH 9.0, where the increase is highest for ATP, followed by ADP (62% compared to ATP) and lowest for AMP (36% compared to ATP) (Fig. 2). Due to the fact that nearly all phosphate is released during the first 15 min at pH 9.0, the differences in slopes may be explained by the availability of phosphate groups with either ATP or ADP or AMP substrate. This hypothesis was confirmed by finding the highest final amount of phosphate using ATP as substrate, followed by ADP and AMP at pH 9.0 (data not shown).

At pH 7.4 the increases in phosphate are considerably slower in case of all substrates compared to pH 9.0 (Fig. 2). However, just as at pH 9.0, most phosphate is released from ATP, followed by ADP and AMP. Although the difference in phosphate increase during the first 30 min is not significant between ATP and ADP substrate at pH 7.4, the mean value was detected to be slightly lower for ADP (86%) compared to ATP (100%), whereas it was

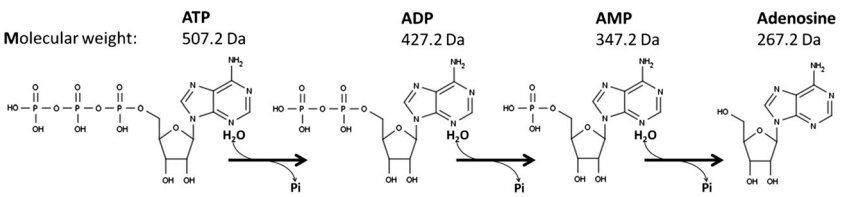

Figure 1. Enzymatic hydrolysis and stepwise removal of inorganic phosphate leads to the generation of the final iAP product adenosine. Nucleotide structures are given along with the average molecular weights of the respective neutral compounds.

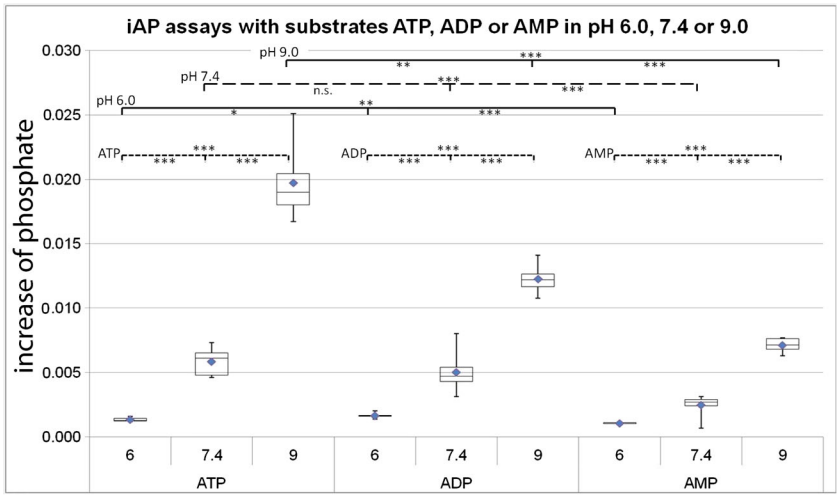

Figure 2. Box-and-whisker plot of numeric values of slopes of linear trend lines of initial phosphate increase photometrically detected; positive and negative whisker, first quartile, median and third quartile, mean value (♦), significant for *p*-values of 0.05 to 0.01 (*), very significant for *p*-values of 0.01 to 0.001 (**) and extremely significant for *p*-values <0.001 (***), not significant for values >0.05 (n.s.), n = 6–9.

significantly reduced with AMP substrate (42%) (Fig. 2). That is because the amount of available phosphate groups does not decrease markedly during the early stages of reaction using ATP or ADP, since the breakdown of ATP or ADP substrate again results in the generation of ADP/AMP or AMP, respectively, which in turn serve as

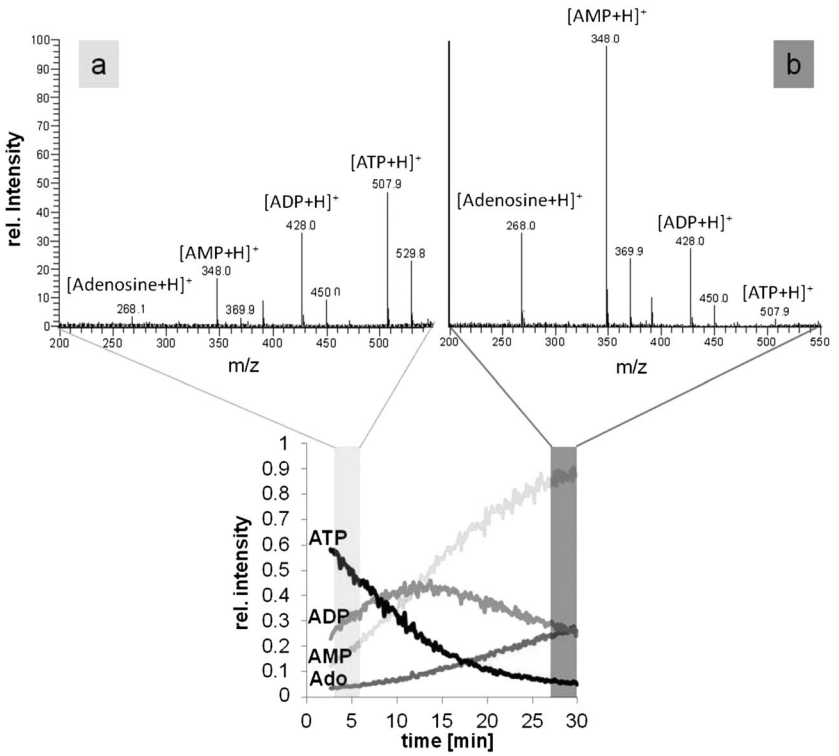

Figure 3. Assay component's signal changes within the *m/z* range 200 to 550 at pH 7.4 at the beginning of enzymatic conversion (a: average intensity of time range minute 3 to 6 corresponding to light grey area) and the end of the measurement (b: average intensity of time range minute 27 to 30 corresponding to dark grey area). Spectrum intensity maximum '100' corresponds to 2.63×10^5 counts. Prominent *m/z* within spectrum: $[Ado+H]^+$: 268.0; $[AMP+H]^+$: 348.0, $[AMP+Na]^+$: 369.9; $[ADP+H]^+$: 428.0, $[ADP+Na]^+$: 450.0; $[ATP+H]^+$: 507.9, $[ATP+Na]^+$: 529.8.

substrate. In contrast with AMP substrate merely the final product adenosine is generated. Therefore, with ATP or ADP compared to AMP substrate, it is more likely for the enzyme to encounter a substrate during the first stages of the reaction, indicating the availability of substrate as the rate-limiting step with AMP substrate at pH 7.4 and ATP, ADP and AMP substrate at pH 9.0.

At pH 6.0 enzymatic activity is distinctly reduced compared to alkaline or neutral pH, resulting in a considerably slower phosphate release from ATP (7% compared to pH 9.0), ADP (13% compared to pH 9.0) and AMP (15% compared to pH 9.0) (Table 1).

However, the differences in slopes (Fig. 2) between the three substrates are significant, with ADP leading to a faster increase in detectable phosphate (122%) than ATP (100%) and AMP (80% compared to ATP). At acidic pH the pyrophosphate synthesis by iAP is enhanced,[26,27] probably leading to an excess production of pyrophosphate with ATP compared to ADP substrate. Since the malachite green assay does not respond to pyrophosphate,[28] its formation would lead to an underestimated phosphate release in the case of ATP substrate. In fact, mass spectrometric measurements (see below) revealed a tendency for faster degradation of ATP compared to that of ADP (83%) at pH 6.0.

Although the application of a colorimetric method to detect the enzymatic activity provides information about the effect of pH on the degradation of different nucleotide substrates, it also reveals several drawbacks. First of all a differentiation between the phosphate released from initial substrate or intermediate products is not achievable. Additionally, it cannot be clarified whether the phosphate groups are removed in a stepwise manner or if, for instance, pyrophosphate is released. This matter was first addressed by Moss *et al.*, who proved a stepwise removal from ATP resulting in ADP and AMP as intermediate products.[15] Nevertheless, this investigation was conducted at one pH-value, i.e. pH 9.5, and solely ATP was used as substrate. So to our best

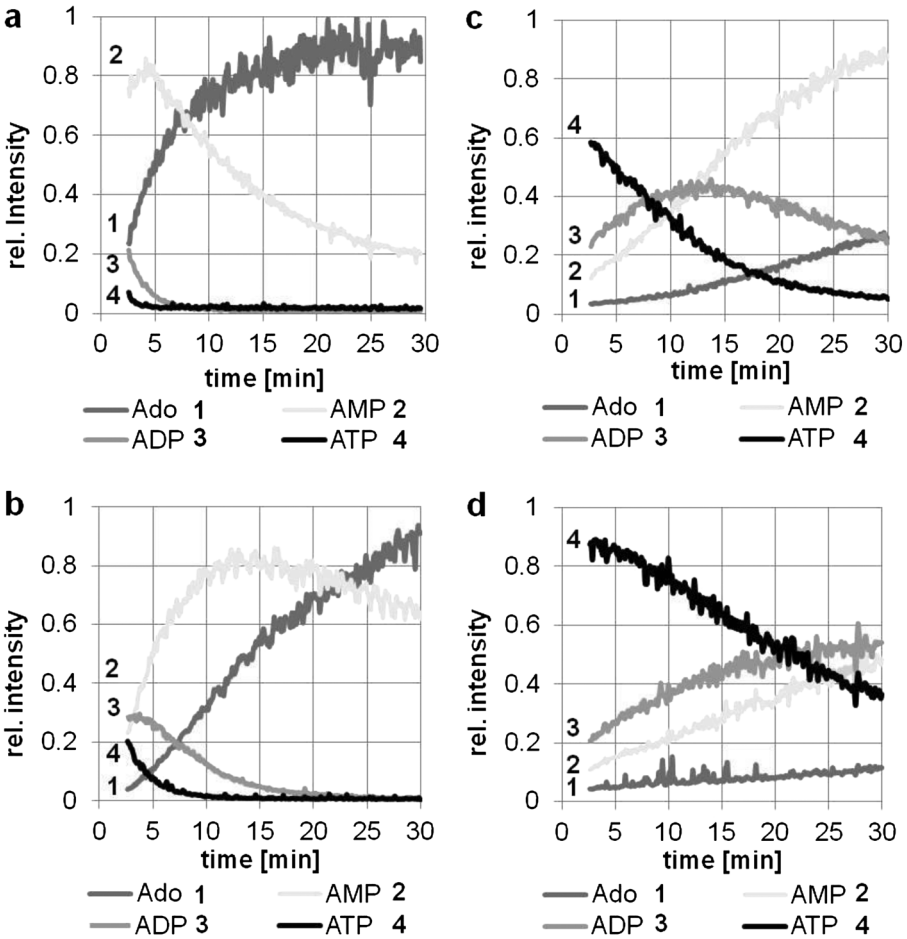

Figure 4. Mass spectrometric determination of enzymatic degradation of 40 µM ATP substrate: (a) iAP concentration 44.64 nM, pH 9.0; (b) iAP concentration 11.16 nM, pH 9.0; (c) iAP concentration 44.64 nM, pH 7.4; (d) iAP concentration 44.64 nM, pH 6.0; *m/z* evaluated, summarized and displayed here: Ado with *m/z* 268.1 and 290.1, i.e. $[Ado+H]^+$ and $[Ado+Na]^+$; AMP with *m/z* 348.1, 370.1, 386.0, 392.0 and 408.0; ADP with *m/z* 428.0, 450.0, 466.0, 472.0 and 488.0; ATP with 508.0, 530.0, 546.0, 552.0 and 568.0, i.e. $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M–H+2Na]^+$ and $[M–H+Na+K]^+$; n = 5–11.

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knowledge, no information about detailed reaction kinetics, the degradation of ADP or AMP as substrates and also about the activity of iAP towards these three substrates at different pH-values is available. For these reasons, a mass spectrometric assay was established to obtain detailed data of the overall course of the reaction. In this regard Fig. 3 exemplarily shows the results of enzymatic ATP conversion at pH 7.4. Traces of ATP substrate, intermediates ADP and AMP and the final product adenosine are presented along with spectra revealing the shift of *m/z* signals associated with the degradation of ATP substrate (*m/z* 507.9, 529.8) and the generation of ADP (*m/z* 428.0, 450.0), AMP (*m/z* 348.0, 369.9) and adenosine (*m/z* 268.1).

MS determination of iAP activity

Applying the same concentrations as in the photometric assay, a nearly complete degradation of ATP within the first 5 min at pH 9.0 was revealed (Fig. 4(a)), that could not be assessed with photometric detection, due to merely detecting the overall phosphate release.

To enable the calculation of conversion rates by applying exponential trend lines to the substrate degradation trace, enzyme concentration at pH 9.0 was quartered to 11.16 nM, resulting in a quartering of enzymatic activity visible by the shift of AMP detection maximum from minute 4.18 with 44.64 nM (Fig. 4(a)) to minute 16.33 with 11.16 nM iAP (Fig. 4(b)). Figure 4 furthermore provides an overview of the ATP substrate degradation, the formation of ADP and AMP intermediates and the generation of the final product adenosine in 10 mM ammonium acetate solution at pH 9.0 (Figs. 4(a) and 4(b)), pH 7.4 (Fig. 4(c)) and pH 6.0 (Fig. 4(d)). In contrast to the photometric approach, for which the slopes of linear trend lines were used to obtain numeric values to enable the comparison of phosphate releases, in the case of MS-detected assays conversion rates were calculated and are presented in Fig. 5.

At pH 9.0 (Fig. 4(a), high enzyme concentration) the degradation of both ATP and ADP is completed within the first 7 to 8 min, whereas AMP slightly increases till minute 5 before also decreasing until minute 30. Furthermore, the AMP curve reveals a flattening, that is also reflected by the curve of the final product adenosine, both indicating a decelerated degradation of AMP at the end of the measurement.

By comparing the substrate degradation at pH 9.0 the conversion rate determined by mass spectrometry is highest for ADP (131%) and lowest for AMP (21% compared to ATP) (Fig. 5 and Table 1). The inferior conversion rate for ATP in comparison to ADP substrate might be associated with an initial substrate competition between ATP and the product ADP and AMP, eventually leading to a decreased degradation and therewith conversion rate of ATP.

Enzymatic assays with ADP substrate revealed a similar progress of fast enzymatic substrate degradation and related intermediate and product generation as those assays measured with ATP substrate (see Supplementary Figs. S2(a)–S2(c), Supporting Information). Like ATP substrate at pH 9.0, ADP substrate is completely degraded within approximately 10 min, revealing a comparable substrate preference for ATP and ADP. In contrast with AMP substrate at pH 9.0 a continuous decrease was observed over the whole measurement time, uncovering AMP as a comparatively poor substrate (21% compared to ATP, Supplementary Fig. S1(a), Supporting Information).

At pH 7.4 the degradation of ATP and simultaneously the generation of ADP are distinctively slower compared to assays conducted at pH 9.0 (Fig. 4(c)). After approximately 12 min a plateau is reached for ADP, i.e. formation from ATP and degradation to AMP is of similar velocity. Contrary to assays at pH 9.0 no decrease in AMP intensity was detected at neutral pH. However, generation of adenosine is apparent during the whole time of measurement, testifying to the degradation of AMP to adenosine, which in conclusion indicates a higher generation of AMP from ADP than degradation of AMP to adenosine.

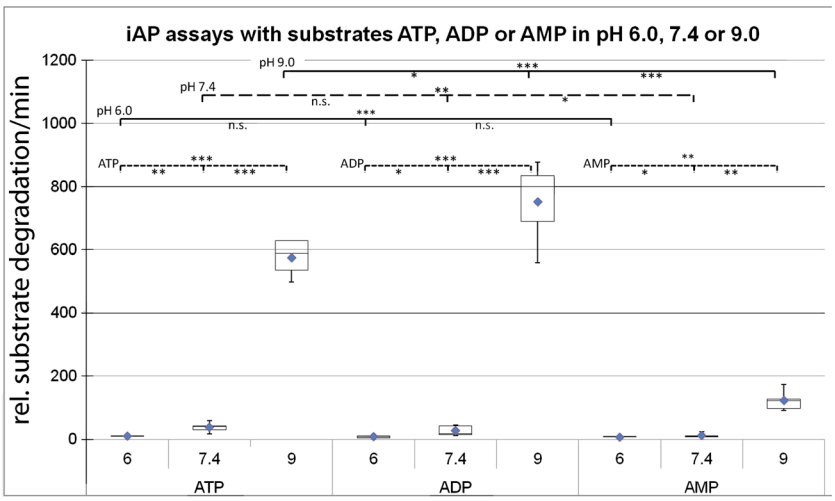

Figure 5. Box-and-whisker plot of substrate conversion rates calculated from exponential trend lines applied to the respective substrate traces mass spectrometrically detected; positive and negative whisker, first quartile, median and third quartile, mean value (◆), significant for *p*-values of 0.05 to 0.01 (*), very significant for *p*-values of 0.01 to 0.001 (**) and extremely significant for *p*-values <0.001 (***), not significant for values >0.05 (n.s.); n = 5–11.

Compared to ATP substrate the conversion rate with ADP substrate at pH 7.4 is decreased to 72% (Fig. 5), pointing out a less pronounced substrate competition at pH 7.4 in contrast to pH 9.0. This is attributed to a lower conversion rate of ATP at pH 7.4 (7%, Fig. 5) compared to the conversion rate of ATP at pH 9.0, resulting in a minor quantity of ADP generated at pH 7.4, which is insufficient to provoke substrate competition. Furthermore, a much more pronounced increase in AMP at pH 9.0 was detected from the beginning of the measurement compared to the increase in AMP at pH 7.4, which results in a higher amount of competitive substrate AMP at pH 9.0.

At pH 6.0 the average conversion rate with ATP substrate was calculated to be even slower (2%) than at pH 7.4 (7%) compared to pH 9.0 (Fig. 5). Nevertheless, an increase in products ADP, AMP and also a slight increase in adenosine was observed (Fig. 4(d)). Average conversion rate at pH 6.0 is highest for ATP, followed by ADP (83%, not significant) and AMP (69%), the latter significantly reduced compared to average ATP substrate conversion rate.

With ADP substrate, conversion rate at pH 6.0 was calculated to be only 1%, whereas at pH 7.4 it is 4% compared to the conversion rate of ADP at pH 9.0. Conversion rates with AMP substrate are 6% at pH 6.0 and 9% at pH 7.4 compared to conversion rate of AMP at pH 9.0. So the relationship between average values of conversion rates for assays at pH 6.0, 7.4 and 9.0 with ADP and AMP substrate is comparable to those obtained with ATP substrate (2% at pH 6.0, 7% at pH 7.4 compared to pH 9.0, Table 1), suggesting a similar response of the enzymatic activity to a decline in pH-value, which is independent of the nucleotide substrate used.

CONCLUSIONS

The mass spectrometric results revealed conversion rates that are considerably lower at pH 7.4 as well as pH 6.0 compared to pH 9.0, which is consistent with the data obtained photometrically (Table 1) and mostly also with data from the literature.[31,32] Furthermore, these findings are in accordance to results of Chappelet-Tordo *et al*. who observed a rise in enzymatic activity and a higher dissociation constant for the enzyme-inorganic phosphate complex with increasing pH-values.[17] Also Cocivera *et al*. demonstrated an increase in activity at alkaline pH and a considerably lower rate constant for the decomposition of the covalent enzyme-inorganic phosphate intermediate at acidic pH-values, thereby causing a lower availability of the enzyme's active site for further breakdown of substrate, indicating this step as rate-limiting.[29] Furthermore, Fernley and Walker demonstrated a decrease in Ki[a] for Pi and PPi at less alkaline pH, suggesting a stronger inhibition of iAP by Pi and PPi at lower pH-values.[30]

However, in contrast to our results, Chappelet-Tordo *et al*.[17] found a considerably higher activity towards AMP substrate compared to ATP substrate at pH 10.0 and a comparable activity at pH 8.0. This indicates a preferred degradation of AMP compared to ATP, although the substrate concentrations of AMP and ATP resulting in maximal velocity (Vm) of substrate conversion detected by Chappelet-Tordo *et al*. are comparable to those applied in this study.

It has been demonstrated in several investigations that diverse factors like type of buffer, ionic strength, metal ions, in particular Mg^{2+} and Zn^{2+}, may influence activity and specificity of iAP.[33,34] For instance, it has been shown that the order of degradation of ATP, ADP and AMP is reversed by addition of magnesium.[31] In this work no cations like Mg^{2+} or Zn^{2+} were added. However, it is unclear whether Chappelet-Tordo *et al*. used any additives, which in turn impedes a final conclusion regarding the disparity of experimental results. Anyhow, they applied buffers containing Tris in the case of pH 8 and ethanolamine at pH 10, both additives being good phosphate acceptors[35] which may result in a lower level of detectable phosphate with ATP.

Moreover, as mentioned before, the 'side reactions' of pyrophosphate formation may also mimic a lower phosphate release from ATP compared to AMP in the measurements of Chappelet-Tordo *et al*. More probably, however, is a difference in the enzyme used. In the study presented here iAP from bovine intestine was employed, whereas Chappelet-Tordo *et al*. used an enzyme originating from calf intestine. This enzyme however was proven in a later study to be heterogenic, i.e. to consist of different forms of the enzyme.[36] These, moreover, possess considerable different activities towards AMP.[37] So it may be assumed that, although a purification step was conducted, a heterogenic mixture of enzymes was used, probably showing markedly different substrate specificity.

In our opinion a lower degradation rate of AMP is more conceivable since the proximity of both the sugar residue and also the base of AMP may cause a steric hindrance or even the formation of interfering hydrogen bridge linkages between the nucleotide base and amino acids of the enzyme active site.

By further comparing the photometric and mass spectrometric measurements at pH 7.4 the results are similar, both revealing the highest enzymatic activity – reflected either by phosphate release or conversion rate – with ATP substrate, followed by ADP substrate (not significant) and AMP substrate (significant). AMP was again detected to be the poorest degradable substrate at pH 6.0, pointing out the clear substrate preference of iAP towards ATP and ADP. Nevertheless, substrate competition between ATP and ADP could be shown only by mass spectrometric detection, due to the ambiguous nature of colorimetric phosphate release detection. Mass spectrometric experiments clearly revealed a higher degradation rate for ADP compared to ATP at pH 9.0, but not at pH 7.4 or 6.0. Furthermore, mass spectrometric detection allowed a detailed investigation of the pH dependence of iAP by elucidating the degradation of substrate, intermediates and the generation of the final product. Increase or decrease of the catalytic activity of iAP due to pH-value shifts is of special interest since iAP takes part in intestinal pH regulation and is furthermore involved in inflammatory processes. In inflammation pH-values tend to become more acidic,[38] thus causing a lowered activity of iAP and therefore a tendency for higher ATP concentration, that would contribute to the progression of inflammatory processes.

[a]Ki = inhibition constant ($Ki = \frac{[E][I]}{[EI]}$) with [E] = concentration of the enzyme; [I] = concentration of the inhibitor and [EI] = concentration of the enzyme–inhibitor complex). Pi = inorganic phosphate and PPi = inorganic pyrophosphate.

Interestingly, the degradation of AMP to adenosine was demonstrated to be distinctively slower, but mainly in very alkaline medium. Nevertheless, a reduced degradation rate of AMP substrate compared to ATP or ADP degradation rates was also observed at physiological pH, although the difference was less pronounced. Physiological consequences in the context of this disparity that may be related to a more outstanding role for AMP in the context of inflammation or pH regulatory processes in the intestinal tract have to be enlightened in the future.

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