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Conductometric Method for the Rapid Characterization of the Substrate Specificity of Amine-Transaminases

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Amine-transaminases (ATAs, ω-transaminases, ω-TA) are PLP-dependent enzymes that catalyze amino group transfer reactions. In contrast to the widespread and well-known amino acid-transaminases, ATAs are able to convert substrates lacking an α-carboxylic functional group. They have gained increased attention because of their potential for the asymmetric synthesis of optically active amines, which are frequently used as building blocks for the preparation of numerous pharmaceuticals. Having already introduced a fast kinetic assay based on the conversion of the model substrate α-methylbenzylamine for the characterization of the amino acceptor specificity, we now report on a kinetic conductivity assay for investigating the amino donor specificity of a given ATA. The course of an ATA-catalyzed reaction can be followed conductometrically since the conducting substrates, a positively charged amine and a negatively charged keto acid, are converted to nonconducting products, a noncharged ketone and a zwitterionic amino acid. The decrease of conductivity for the investigated reaction systems were determined to be 33−52 μS mM^{-1}. In contrast to other ATA-assays previously described, with this approach all transamination reactions between any amine and any keto acid can be monitored without the need for an additional enzyme or staining solutions. The assay was used for the characterization of a ATA from *Rhodobacter sphaeroides*, and the data obtained were in excellent agreement with gas chromatography analysis.

The identification of novel biocatalysts and the optimization of existing enzymes are key steps for developing highly efficient processes. Thus, protein engineering and the metagenome approach have emerged as powerful tools that are used frequently to achieve these aims. While working with large enzyme libraries which are generated during these strategies, the fast, effective, and reliable identification of enzymes with the desired properties is often the bottleneck since this is the most time-consuming step. Therefore a large variety of fast methods for determining enzyme activity have been developed for many biocatalysts, which often are capable of being performed in a high-throughput screening (HTS) format. Examples includes solid-phase bound assays related to immunoassay,[1] IR thermography,[2] circular dichroism (CD) spectroscopy,[3,4] fluorescence image analysis,[5] and UV−vis-based methods.[6,7]

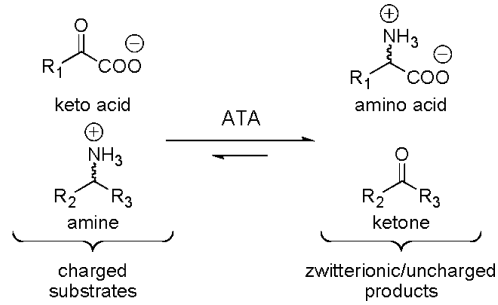

Figure 1. Transaminase reaction and principle of the conductivity assay. A positively charged amine and a negatively charged keto acid are converted to a zwitterionic amino acid and a noncharged ketone, thus the conductivity of the reaction solution decreases.

Amine-transaminases (ATA, ω-transaminases, ω-TA) gained increased attention in research in the last years because of their potential for the synthesis of optically active amines, which are frequently used as building blocks for the preparation of numerous physiologically active compounds. However, for the screening of the substrate specificities and enantioselectivities of ATA only a limited number of methods have been reported, due to the special type of reaction catalyzed (Figure 1): An amine and a carbonyl substrate bearing a ketone or aldehyde group are converted into products by exchanging their amino and carbonyl functional groups, without the stochiometric consumption of a cofactor. In case of other enzymatic reactions like e.g. hydrolysis of esters or amides, the substrates (ester/amide) and products (alcohol/amine

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Table 1. Calibration of the Conductivity Assay[a]

substrate/product	**1** & **6**	**2** & **6**	**3** & **6**	**4** & **6**	**5** & **6**	**3** & **7**	**3** & **8**	**3** & **9**	**3** & **10**
conductivity [μs mM^{-1} cm^{-1}]	36.6	39.0	44.0	34.6	32.9	37.1	40.0	49.5	52.1

[a] The values reflect the change in conductivity due to the conversion of 1 mM substrates to products (see Figure 4 for structures of substrates and products **1**-**10**). The resolution of the applied conductometer was 0.1 μS.

and acid) can be discriminated very easily,[8] or during reactions involving a cofactor like NAD(P)H or side product like H_2O_2, these compounds can be detected specifically.[9,10] For ATA, this is not the case, and there are only a few special methods to discriminate the similar substrates and products without affecting enzyme activity.[11]

In addition to ketones and aldehydes, ATA also convert the α-keto carboxylates pyruvate and glyoxylate. The methods for assaying aminotransferase activity published so far comprise (i) measurement of the generated amino acid (alanine, glycine) via detection of the corresponding copper complex[11] and (ii) a pH indicator based multienzyme cascade assay.[12] Obviously, there are several drawbacks: the copper staining solution in method (i) inhibits the enzyme, and nonspecific color formation with several buffers and crude cell extracts are observed. Thus, only end point measurements are possible, and appropriate measurements of the blank are necessary. Method (ii) works in the asymmetric synthesis mode (Figure 1, reaction from right to left for bottom partners), and thus no information about enantiopreference or -selectivity can be obtained.

Alternatively, we recently published an UV-spectrophotometric assay based on the conversion of the widely used model substrate α-methylbenzylamine.[13] The product from this reaction, acetophenone, can be detected spectrophotometrically at 245 nm with high sensitivity (ε = 12 mM^{-1} cm^{-1}), since the other reactants showed only a low absorbance. Besides the standard substrate pyruvate, all low-absorbing ketones, aldehydes, or α-keto carboxylates could be used as cosubstrates. Thus, the assay is a fast and easy method for determining transaminase activity. Additionally, the amino acceptor specificity of a given ATA can be characterized very quickly.

As the acetophenone assay is limited to α-methylbenzylamine as amino donor, we now developed a method for a fast and easy characterization of the amine donor specificity of a given ATA. With this conductivity based approach every amine donor and acceptor can be applied as substrate, as long as one of the substrates is an amino or keto acid, respectively.

EXPERIMENTAL SECTION

All conductivity mesasurements were performed using either a Qcond 2200 (VWR, Germany) or a CX-401 (Elmetron, Poland) multimeter with a graphite (∅ 12 mm) or a platinum (∅ 5 mm) electrode, respectively.

Buffer Preparation. All buffers had a concentration of 20 mM and were adjusted to pH 7.5. Tris was adjusted with HCl, the three buffers containing BES, CHES, and HEPES were adjusted with Bis-Tris (bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane), and EPPS and tricine were adjusted with 1,8-diazabicyclo[5.4.0]-undec-7-en.

Calibration. For calibrating the assay, different standard solutions with decreasing substrate and increasing product concentrations of 0−10 mM were prepared, and the conductivity and pH were measured (Table 1). No change of the pH could be detected. It is noteworthy that the accuracy of the assay was significantly higher starting from 10 mM substrates than starting from 5 mM substrates. Furthermore, it was important to use all reactants simultaneously, including the nonconducting ketone and alanine, for the calibration (see results). For analyzing the influence of crude extract on the conductivity measurements, standard curves with different amounts (0−18% (v/v)) of crude extract (OD_{600} ≈ 10) of *E. coli* BL21 without ATA were measured in parallel. For the preparation of the crude extract, cells were washed twice and disrupted by sonication in the buffer used for conductivity measurements.

Kinetic Measurements. The reactions were carried out at room temperature (RT). Kinetic measurements were performed in reaction mixtures containing 10 mM substrates in buffer (20 mM, pH 7.5), 10% dimethyl sulfoxide, and an appropriate amount of enzyme (purified enzyme or crude extract). Using the platinum (∅ 5 mm) electrode, the reaction can be carried out in a well of a microtiter plate, and a reaction volume of 200 μL is sufficient. The course of the reaction was followed by measuring the conductivity of the solution continuously. Each measurement was repeated at least three times. For blank measurement, the reaction was carried out with cell extract lacking an amine-transaminase or with reaction mixtures containing only one of the substrates, but no significant change in conductivity could be detected. The specific activity was expressed as units per milligram protein. One unit of activity was defined as the amount of enzyme that produced 1 μmol ketone product per minute.

Validation of the Assay with Gas Chromatography. During kinetic measurement, aliquots were taken at certain points for validation of the conductivity assay by gas chromatography. The reaction was stopped by adding 1/10 of the volume of 1 M HCl. The ketones formed during the reaction were extracted with one volume of ethyl acetate and determined by gas chromatography (Hewlett-Packard 5890 Series II Plus) using a forte BP-21 column (SGE, Griesheim, Germany) with benzaldehyde as internal standard.

Characterization of the ATA from *Rhodobacter sphaeroides*. The ATA from *Rhodobacter sphaeroides* 2.4.1 (DSM 158) was recombinantly expressed in *E. coli* BL21 (DE3) and purified via

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Table 2. Summary of the Buffer Systems Investigated[c]

buffer[a]	pK_a	conductivity [μS cm^{-1}]	rel activity [%][b]
PB	7.2	2760	100
BES	7.3	445	120
HEPES	7.7	141	24
EPPS	8.0	246	76
Tris	8.1	1600	71
tricine	8.3	240	98
CHES	9.4	46	67
A. dest	−	3	0

[a] PB: phosphate buffer; BES: *N*,*N*-bis(2-hydroxyethyl)taurine; HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EPPS: 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Tris: 2-amino-2-(hydroxymethyl)-1,3-propanediol; tricine: *N*-[tris(hydroxymethyl)methyl]glycine; CHES: 2-(cyclohexylamino)ethanesulfonic acid. [b] Activities in the different buffer systems were determined using the acetophenone assay.[13] [c] All buffers had a concentration of 20 mM and were adjusted to pH 7.5 (see the Experimental Section).

affinity chromatography (IMAC sepharose) as reported previously.[13]

Protein concentrations were determined using the BCA assay kit (Uptima, Montlucon, France).

To determine the substrate specificity of the enzyme, 10 mM of different substrates and an appropriate amount of purified enzyme (0.1−2 mg mL^{-1}) were mixed with 1 mL of 20 mM tricine buffer pH 7.5. Dimethyl sulfoxide (10% (v/v)) was added to the mixture to dissolve hydrophobic substrates. Solutions of oxaloacetate and α-ketoglutarate were prepared freshly prior to measurements. The reaction was carried out at RT and followed by measuring the conductivity continuously. For validation by GC, aliquots were taken at certain points, and the concentration of the ketone products **1b**−**5b** was analyzed as described above.

RESULTS AND DISCUSSION

In the course of an ATA-catalyzed reaction the conductivity of the reaction medium changes since charged reactants (amine and keto acid) are converted to noncharged species (the ketone and a zwitterionic amino acid) (Figure 1). For all experiments, the transamination was carried out in kinetic resolution mode. The rationale behind this approach is as follows: (i) the reaction equilibrium favors product formation, (ii) both enantiomers of a given amine substrate can be used separately in the assay, so that also information about enantioselectivity can be obtained, and (iii) the amine substrate usually is much better soluble than the corresponding ketone. Thus, even with higher substrate concentrations a homogeneous assay solution is obtained rather than a biphasic system, which otherwise might interfere with the measurements.

This allows a simple measurement of the reaction progress, if some crucial requirements are fulfilled: First, to maximize sensitivity the buffer system should have low background conductivity. The pH of the buffer has to be kept in a pH range from 4.0−8.0, where the net charge of alanine is ≈0. Furthermore, for practical reasons or for high-throughput screening purposes, the assay should not be sensitive toward variations in crude extract concentrations.

Standard buffer systems like phosphate buffer or Tris-HCl could not be applied due to their very high conductivity, whereby the relative change in conductivity caused by the enzymatic reaction is rather small. Thus, Good's buffers were investigated regarding the conductivity of 20 mM solutions at pH 7.5 and effects on aminotransferase activity (Table 2). Due to their zwitterionic nature, solutions of these compounds show a much smaller conductivity if the pH is kept within 2 units near the pI of the respective compound (isoelectric buffers).

The first system investigated was BES-Bis-Tris. It showed a very good performance with purified enzyme, but unfortunately activities of crude extract measured in this buffer did not match the results obtained by gas chromatography. Although the CHES-Bis-Tris system showed excellent performance with both purified enzyme and crude extract, due to its inhibitory effect (67% enzyme activity compared to phosphate buffer) and its rather low buffer capacity at pH 7.5, we explored an alternative buffer system. The inhibitory effect was the same for the EPPS buffer (76% rel activity) and even greater for the HEPES-Bis-tris system (24% rel activity). Finally, the tricine buffer showed both a good buffer capacity at pH 7.5 and a very good agreement of determined activities of purified enzyme and crude extract by measuring the conductivity compared to GC analysis (Figure 2). For this reason the tricine buffer was used for all subsequent experiments. The inhibitory effect of the EPPS and CHES buffer may vary for other transami-

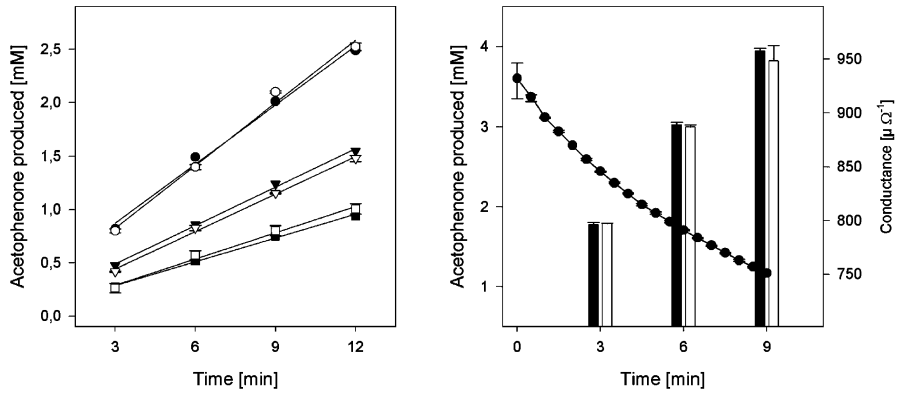

Figure 2. Validation of the assay by following the reaction of 10 mM (*S*)-α-methylbenzylamine and pyruvate using both conductivity measurements (black symbols/bar) and detection of the acetophenone formed by gas chromatography (white symbols/bar). The calculated activities differed only by 5% for purified enzyme (left, □ 0.25 mg/mL, ▽ 0.5 mg/mL, ○ 1 mg/mL) and crude extract (10% (v/v)) (right, the curve displays the conductivity measurement, the bars show the calculated acetophenone concentrations).

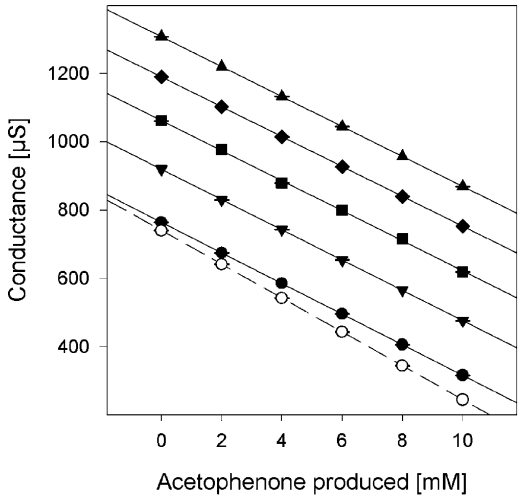

Figure 3. Influence of different concentrations of crude extract (● 0%, ▼ 5%, ■ 10%, ◆ 14%, ▲ 18% (v/v)) on the change of conductivity during the transaminase reaction. While measuring the standard curves all reactants have to be present, as if measured separately, the calculated rate $\Delta\mu S\ mM^{-1}$ (○) is significantly higher. Contrary to other buffer systems investigated (and at substrate concentrations of 5 mM), no significant influence of different crude extract concentrations were observed.

nases, so these buffers should be considered for further experiments.

In practice and especially for screening of many samples, crude cell extract is used as enzyme preparation instead of purified proteins. Thus an important question was whether the presence of different amounts of crude extract has an impact on the conductivity measurement. As expected, the background conductivity of the reaction medium increased proportional to the concentration of cell extract used. Next, calibration experiments were performed where different conversions were simulated by varying the concentrations of all reactants according to a real reaction. Fortunately, the addition of different amounts of crude extract did not seem to affect the change of conductivity significantly (Figure 3). This was surprising, because in theory the molar conductivity of a given compound depends on the concentration of all electrolytes present in the reaction system for mainly two reasons: on the one hand, the protonation state and thus the net charge of an analyte may be affected by other compounds present in the solution. On the other hand, more importantly, ions or even neutral molecules from the background electrolyte may affect the mobility and thus the molar conductivity of an analyte significantly by intermolecular electrostatic or hydrophobic interactions. This explains why the conductivity of a mixture comprised of different ions usually differs to some extent from the sum of the contributions of the single ions to overall conductivity. For this reason, all participating reactants and even the neutral species had to be included in the calibration of the assay. If otherwise the change of the conductivity per milimolar reaction conversion [μS/mM] is calculated from molar conductivities of the single compounds, the obtained value would be higher (49.4 μS/mM conversion, ○ in Figure 3) compared with the measurement of the complete mixtures (44.1 μS/mM conversion, ● in Figure 3).

Application of the Assay in the Characterization of an ATA from *Rhodobacter sphaeroides*. To verify the method described above, an ATA identified in the genome of *Rhodobacter sphaeroides* 2.4.1 was characterized using this assay. The enzyme was expressed recombinantly in *E. coli* BL21 and purified as described.[13] Investigation of the amino donor profile revealed that besides the model substrate α-methylbenzylamine also benzylamine is a very well accepted substrate (Figure 4). Aliphatic and arylaliphatic compounds were converted only slowly, especially the cyclic aliphatic amine 1-N-Boc-3-aminopiperidine. The results obtained for the amino acceptor profile are in good agreement with previously measured data using the acetophenone assay.[13] Pyruvate is the best converted acceptor, glyoxylate and succinic semialdehyde are converted moderately, and the keto-dicarboxylic acids oxalacetate and α-ketoglutarate are converted only very slowly.

The conductivity assay is an excellent complementary method to the acetophenone assay, since the usually more interesting amino donor specificity of an ATA can be investigated. With both assays in hand, the complete characterization of the substrate specificity can be performed very rapidly. Furthermore, the conductivity assay allows a fast screening of ATA variants for the reaction of any desired amine with a keto acid. One limitation of the conductivity assay is that an appropriate low conductivity buffer

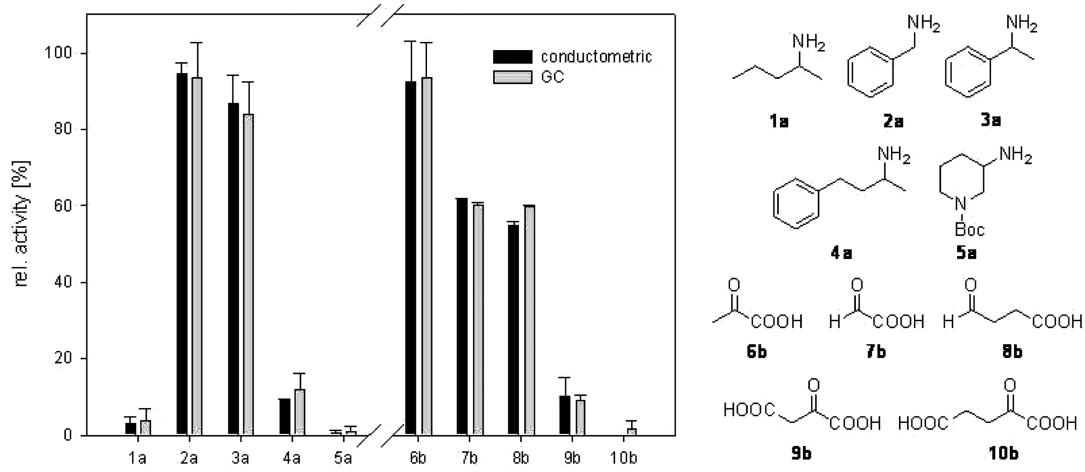

Figure 4. Substrate specificity of *Rhodobacter sphaeroides* ATA was determined using the conductivity assay (black) as well as gas chromatography (gray). All measurements were repeated three times.

in the pH-range of 4−8 has to be used. For determining other enzymatic properties like the pH- or temperature optimum of an ATA, the acetophenone assay is more flexible since every low absorbing buffer at any pH may be used and absorbance is virtually not dependent on temperature in contrast to conductivity, so that multiple calibrations can be avoided.

CONCLUSIONS

In this contribution we described a simple and sensitive conductivity assay that can be used for the fast characterization of the amino donor substrate profile of ATA. This was achieved without the need for any additional enzymes or staining solutions. With appropriate equipment, we envision an application of this assay as a high-throughput method for screening enzyme libraries.

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