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An accurate method for estimation of the intracellular aqueous volume of *Escherichia coli* cells

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ABSTRACT

The intracellular aqueous volumes (V_{in}) of microorganisms are fundamental data that can be used for estimating absolute cellular enzyme and metabolite concentrations. Because traditional methods are time–consuming and costly, the V_{in} data have been largely estimated ambiguously. Here we developed an NAD^+ concentration-dependent method and demonstrated its usefulness for accurate estimation of the V_{in} value of *Escherichia coli* cells. The V_{in} value of *E. coli* BL21(DE3) cells was determined to be 1.9 $\mu L \cdot mg^{-1}$, which is 17% lower than that of the commonly assumed data. Similarly, the V_{in} value of *Agrobacterium tumefaciens* AGL1 cells was determined to be 1.8 $\mu L \cdot mg^{-1}$. Because NAD^+ is routinely quantified during metabolite analysis, it may be integrated into metabolomic data collection with little additional time and labor expenditure. This method should also be applicable to estimate the V_{in} data of other prokaryotic microorganisms.

1. Introduction

Absolute enzyme and metabolite concentrations are fundamental data for the development of reliable metabolic models and rational engineering of industrial microorganisms, as concentrations affect both thermodynamics and dynamics of cellular processes (Bennett et al., 2009; Schomburg, 2009). While many methods have been developed to determine concentrations of cellular components in a sample with high accuracy (Bennett et al., 2008; Tredwell et al., 2011), it is pivotal to measure the intracellular aqueous volume (V_{in}) precisely, such that absolute concentrations of those cellular components can be reached. However, the V_{in} values have been estimated in an ambiguous way in many cases. For example, the V_{in} value has been approximated as 2.3 μL per mg of dry cell weight (DCW), or more roughly, referred as the volume of the whole cell (Bennett et al., 2008; Brown and Dunn, 1989; Loferer–Krossbacher et al., 1998). A number of weight- or concentration-dependent methods have been known for the estimation of the V_{in} value. Thermogravimetrical method is considered as a quick weight-dependent one for the estimation of intra- and extracellular water of microbial cells (Illmer et al., 1999). This method uses expensive instrument, and usually leads to over-estimation of the intracellular water because of complications by other volatile components. Nuclear magnetic resonance-based, concentration-dependent methods have also been developed to determine the V_{in} value, but those methods require abiotic, isotope-labeled substances as probes (Hoffman and Gupta, 1986; Cayley et al., 1991; Quiros and Salas, 1996).

Here we developed an NAD^+ concentration-dependent method for accurate determination of the V_{in} value and demonstrated its usefulness for *Escherichia coli* and *Agrobacterium tumefaciens* cells. NAD^+ was chosen as the probe because it can significantly affect the metabolic networks and has been routinely quantified during metabolic data collection (Roca et al., 2003; San et al., 2002). Furthermore, because prokaryotic cells are free of intracellular compartmentalization, the V_{in} data can be readily used for the estimation of absolute concentrations of cellular components (Bennett et al., 2009; Rabinowitz, 2007). This method is relatively simple in terms of attaining accurate V_{in} data because it avoided interferences by other volatile substances.

2. Material and methods

2.1. Strains and reagents

E. coli BL21(DE3) and NAD^+ were purchased from Dingguo Biotech. (Beijing, China). *A. tumefaciens* AGL1 was kindly provided by Prof. Xiaofeng Dai of Institute of Crop Science, Chinese Academy of Agricultural Sciences. Tryptone and yeast extracts were supplied by Thermo Scientific. Acetonitrile (HPLC grade) was purchased from Merck. Ammonium acetate and other chemicals were purchased from Sigma.

2.2. Cell cultivation and cell pellets preparation

Cells were routinely grown aerobically in LB medium (10 g tryptone, 5 g yeast extracts, 10 g NaCl per liter water) at 37 °C for *E. coli*

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BL21(DE3) and 30 °C for *A. tumefaciens* AGL1 with agitation at 200 rpm on an HZQ-QX type orbital shaking incubator (Harbin Donglian Electronics Co., Harbin, China). Strains were picked into 5 mL of LB medium, cultivated for 12 h, and then inoculated into 250 mL of LB medium for additional 12 h. Cells were harvested by centrifugation on a himac CF16RX type centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan) for 6 min at 2000 ×*g*, 4 °C, washed with 100 mL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.0) twice and resuspended in 150 mL of PBS. The suspension was carefully divided into 6 mL aliquots, and cell pellets were collected by centrifugation for 6 min at 2000 ×*g*. Cell pellets were also collected and lyophilized to a constant weight to determine DCW.

2.3. Procedures for determination of the V_{ex} value

To determine the V_{ex} value, a brief procedure is as follows, 1) prepare cell pellet sample as described above; 2) prepare standard NAD^+ solution [NAD] in PBS buffer; 3) mix V_{NAD} amount of the standard NAD^+ solution with cell pellets; 4) centrifuge for 6 min at 2000 ×*g* and quantify the diluted NAD^+ concentration [NAD_1]; 5) plot [NAD_1] against [NAD] and identify the slope k_1; and 6) calculate the V_{ex} value according to Eq. (4) shown in Fig. 1. In a typical experiment, V_{NAD} was 400 μL, and the concentrations of standard NAD^+ solution were 0, 1, 2, 3 and 4 mM.

2.4. Procedures for determination of the V_{in} value

To determine the V_{in} value, a brief procedure is as follows, 1) prepare cell pellet sample as described above; 2) prepare standard NAD^+ solution [NAD] in 0.2 M HCl; 3) mix V_{NAD} amount of the standard NAD^+ solution with cell pellets; 4) release intracellular NAD^+ by mixing with 450 μL of chloroform pre-cooled at −20 °C, treating twice by the process of freezing in liquid nitrogen and thawing at 50 °C for 10 min; 5) centrifuge for 15 min at 15,000 ×*g*, neutralize the supernatant with equal volume of 0.1 M NaOH and quantify the diluted NAD^+ concentration [NAD_2]; 6) plot [NAD_2] against [NAD] and identify the slope k_2; and 7) calculate the V_{in} value according to Eq. (8) shown in Fig. 1. In a typical experiment, V_{NAD} was 400 μL, and the concentrations of standard NAD^+ solution were 0, 1, 2, 3 and 4 mM.

2.5. Determination of the V_{in} value in combination with gravimetrical method

The V_{in} value was also estimated by subtracting the V_{ex} value, determined by this method, from the total water volume (V_{total}). The V_{total} value was estimated gravimetrically upon the cell pellets being dried by lyophilization.

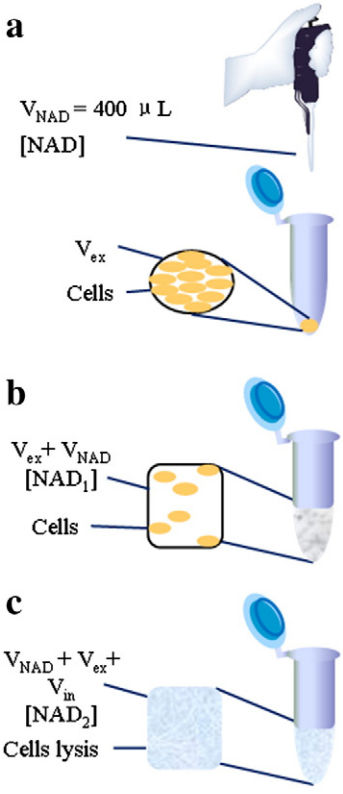
2.6. NAD^+ quantification

An HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Dionex P680A LPG pump, UVD170U UV detector, and a SinoChrom ODS-BP column (4.6 by 200 mm, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China), was used for the determination of NAD^+ concentration as described previously (Sporty et al., 2008). The rate of the mobile phase (95% 50 mM ammonium acetate and 5% acetonitrile) was set at 1 mL/min, and the detector was set at 260 nm.

3. Results

3.1. Determining the V_{ex} value by using NAD^+ as the probe

The total aqueous volume of wet cell pellets is consisted of extra- and intracellular water. Although the total aqueous volume can be



$$V_{NAD} \times [NAD] = (V_{ex} + V_{NAD}) \times [NAD_1] \quad (1)$$

$$[NAD_1] = \frac{V_{NAD}}{V_{ex} + V_{NAD}} \times [NAD] \quad (2)$$

$$k_1 = \frac{V_{NAD}}{V_{ex} + V_{NAD}} \quad (3)$$

$$\mathbf{V_{ex} = V_{NAD} \times (1 - k_1)/k_1} \quad (4)$$

$$V_{NAD} \times [NAD] + V_{in} \times [NAD_{in}] = (V_{NAD} + V_{ex} + V_{in}) \times [NAD_2] \quad (5)$$

$$[NAD_2] = \frac{V_{NAD}}{V_{NAD} + V_{ex} + V_{in}} \times [NAD] + \frac{V_{in}}{V_{NAD} + V_{ex} + V_{in}} \times [NAD_{in}] \quad (6)$$

$$k_2 = \frac{V_{NAD}}{(V_{NAD} + V_{ex}) + V_{in}} = \frac{V_{NAD}}{V_{NAD}/k_1 + V_{in}} \quad (7)$$

$$\mathbf{V_{in} = V_{NAD} \times \frac{k_1 - k_2}{k_1 \times k_2}} \quad (8)$$

Fig. 1. Procedures and principle for estimating the intracellular water volume. Step a, sample preparation; step b, dilution of the probe solution by extracellular water; step c, dilution of the probe solution by intra- and extracellular water. Detailed equations are included to illustrate the principle. [NAD]: concentration of the probe NAD^+ solution, V_{NAD}: the volume of the probe NAD^+ solution, V_{ex}: volume of extracellular water, V_{in}: volume of intracellular water, [NAD_1]: observed NAD^+ concentration after dilution by extracellular water, [NAD_2]: observed NAD^+ concentration after dilution by intra- and extracellular water.