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A Protein Biosensor for Lactate

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Abstract

Blood lactate is a clinically valuable diagnostic indicator. In this preliminary report we describe a protein biosensor for L-lactate based on beef heart lactate dehydrogenase (LDH). LDH was noncovalently labeled with 8-anilino-1-naphthalene sulfonic acid (ANS). The ANS-labeled LDH displayed an approximately 40% decrease in emission intensity upon binding lactate. This decrease can be used to measure the lactate concentration. The ANS-labeled LDH was further utilized in a new sensing format, polarization sensing, which is suitable for miniaturization to a point-of-care lactate monitor. However, temporal instability of beef heart LDH indicates the need for further protein engineering prior to development of a more robust lactate-sensing protein.

There is considerable medical interest in measurements of blood lactate. A resting adult produces approximately 1.3 mol of L-lactate per day. L-Lactate, referred to here as lactate, is removed by gluconeogenesis in the liver and to a smaller extent by oxidation in skeletal muscle and the renal cortex (1). Normal resting concentrations of blood lactate range from 0.36 to 0.75 mM at rest with somewhat higher values of 0.36 to 1.7 mM for hospitalized patients (1). Elevated concentrations of blood lactate are indicators of a considerable number of medical conditions. As examples, serum lactate levels are predictive of survival in children after open heart surgery (2) and mortality in ventilated infants (3) and may be preferable to pH for evaluating fetal intrapartum asphyxia (4). In adults elevated blood lactate can predict multiple organ failure and death in patients with septic shock (5) and the function of newly transplanted livers (6). Lactic acidosis is also known to accompany decreased tissue oxygenation, hypovolemic, left ventricular failure, and drug toxicity (1). Measurement of blood lactate is also valuable for monitoring the results of exercise and athletic performance (7). D-Lactate is not produced by humans, and is found in the blood except in the presence of unusual intestinal bacteria.

While blood lactate is a useful diagnostic indicator, its use is hindered by the time required for a lactate determination. Even under favorable conditions a lactate measurement typically takes 30 min or longer, which is too long for many clinical decisions, particularly with critically ill patients (8). Lactate determinations are typically performed by enzymatic oxidation to pyruvate by lactate dehydrogenase (LDH)³ or lactate oxidase, followed by detection of NADH or H₂O₂, respectively (1, 9–11).

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In the present report we describe an alternative method for measuring lactate using LDH. LDH is a tetramer with a molecular weight of $136,700 \pm 2100$ Da. A number of isozymes are known to occur as mixed tetramers of the muscle and heart isozymes. We demonstrated that beef heart LDH, when noncovalently labeled with 8-anilino-1-naphthalene sulfonic acid (ANS), displays a decrease in the ANS emission intensity upon binding lactate. This decrease in the ANS fluorescence occurs without consumption of lactate. Additionally, the changes in intensity were shown to be measurable with a simple device which could be developed for use at the patients' bedside.

MATERIALS AND METHODS

Beef heart LDH, ANS, and L-lactate were obtained from Sigma. LDH was extensively dialyzed against 10 mM sodium phosphate buffer, pH 6.0, at 4°C. After dialysis the enzyme solution was centrifuged at 12,000 rpm for 30 min at 4°C and the supernatant was recovered. The supernatant was filtered by utilizing an inorganic membrane filter, Anotop 10 (Whatman). The obtained enzyme solution represents the starting material for the fluorescence measurements. For all fluorescence measurements the final concentrations of ANS and LDH were 4 and 3 μM , respectively. Steady-state fluorescence measurements were performed in quartz cuvettes in an ISS spectrofluorometer using magic angle polarizer conditions.

Frequency-domain (FD) measurements were performed using instrumentation described previously (12, 13). For excitation at 295 nm the light source was a frequency-doubled cavity dumped R6G dye laser, and the emission observed through a 340-nm interference filter. For 370-nm excitation the light source was a pyridene 2 dye laser and the emission observed through a 465-nm interference filter. The FD measurements were also performed using magic angle polarizer conditions.

The FD intensity decay data were analyzed by nonlinear least squares in terms of the multiexponential model

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad [1]$$

where α_i is the preexponential factor associated with the decay time τ_i , with $\sum_i \alpha_i = 1.0$. The mean lifetime is given by

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i, \quad [2]$$

where f_i is the fractional steady-state intensity of each lifetime

³Abbreviations used: ANS, 8-anilino-1-naphthalene sulfonic acid; FD, frequency domain; LED, light-emitting diode; LDH, beef heart lactate dehydrogenase.

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}. \quad [3]$$

The amplitude-weighted lifetime is given by

$$\langle \tau \rangle = \sum \alpha_i \tau_i. \quad [4]$$

The values of $\langle \tau \rangle$ are thought to be proportional to the quantum yield of the sample.

A brief description of the theory for polarization sensing is given in the Appendix.

RESULTS

Figure 1 shows the intrinsic tryptophan emission of LDH. Addition of micromolar concentrations of lactate resulted in an approximately 30% decrease in the tryptophan intensity, consistent with an earlier report on the C₄ isozyme of LDH (14). Since the intensity remained rather constant upon addition of more lactate, we believe that the enzyme is mostly saturated by 200 μ M lactate. We questioned whether lactate binding affected the lifetime or intensity decay of LDH. The frequency-domain intensity decays are shown in Fig. 2, and the multiexponential analyses of these data are given in Table 1. We found no significant change in the mean lifetime, intensity-weighted lifetime, or intensity decay due to lactate binding. Given the modest change in the emission intensity, and the difficulty of using intrinsic protein fluorescence in a clinical setting, we concluded the intrinsic emission of LDH is not useful for lactate sensing.

A clinically useful sensor requires that the wavelength be long enough to allow the use of simple excitation sources. Within the past several years UV output near 370 nm has become available from light-emitting diodes (LED) (15–17), and laser diodes as short as 399 nm have been reported (18, 19). LEDs are also known to be useful for nanosecond lifetime measurements because of the capability of high-frequency modulation (16, 17, 20, 21). Hence we labeled LDH with a fluorophore suitable for LED excitation. As a first step we chose ANS because of its well-known sensitivity to its local environment. We found that the emission intensity of an ANS solution with LDH was about 30-fold higher and displayed a blue shift from 525 to 465 nm. ANS concentrations significantly higher than the LDH concentration did not appear to bind to LDH under our experiment conditions. This result is consistent with previous reports that tetrameric LDH binds 4 to 6 ANS molecules, and further addition of ANS does not result in further ANS binding (22, 23). Importantly, the emission intensity of the LDH-bound ANS was sensitive to lactate, and decreased about 40% upon lactate binding (Figs. 3 and 4). We also found that the mean lifetime of ANS decreased upon binding of lactate (Fig. 5 and Table 2). However, these lifetime changes were rather modest and were judged to be too small for lifetime-based sensing (24).

In recent publications we addressed the problem of obtaining reliable intensity measurements for sensing which could be used in the absence of useful changes in lifetimes. We developed two such methods, modulation sensing (25, 26) and polarization sensing (27, 28). Polarization sensing is accomplished by constructing a sensor such that a stable

intensity reference is observed through one polarizer and the sample is observed through a second orthogonal polarizer. One such configuration is shown in Fig. 6. In this case the reference is an ANS-LDH solution in the absence of lactate, which can be expected to display similar temperature-, time-, or illumination-dependent changes as the sample. This reference is observed through a vertically oriented polarizer. The sample contains ANS-LDH and various concentrations of lactate, and is observed through a horizontally oriented polarizer. The emission from both sides of the sensor is then observed through an analyzer polarizer. The analyzer polarizer is rotated until the emission from both sides is equalized, which can be measured visually (28) or with a simple photocell or photodiode circuit shown in Fig. 6 (29). In this case the analyzer polarizer is rotated until the voltage across the differential electronics (Watson bridge) is zero. The angle of polarizer rotation can then be used to determine the lactate concentration. We called this angle the “compensation angle.” A more complete description of polarization sensing is given in the Appendix.

In the previous publications we calculated the change in compensation angle expected for various changes in sample intensity. The simulations in Fig. 6 are for intensity changes comparably to those observed for ANS-LDH. The lower panel of the Fig. 6 shows the compensation angle dependence, α from different initial ratio of reference to sample fluorescence intensity, $k = I_R/I_S$. Series of curves are plotted for different values of n which represents expected total intensity change of sensing fluorophore in response to analyte. These simulations show that a 40% change in intensity is only expected to result in a modest 6° change in the compensation angle.

We used the apparatus in Fig. 6, with a dual photodiode detector, to measure lactate concentrations (Fig. 7). The intensity change induced by analyte (lactate) results in a change of the compensation angle, α , which is related to the concentration of analyte (28, 29). As predicted from Fig. 6, for total intensity change $\sim 40\%$ ($n \approx 1.7$) the change in compensation angle was about 6° for the entire range of lactate concentrations. While the range seems small, the compensation angles are readily measured to about 0.1° so that a 6° change corresponds to an accuracy of 2% in the lactate concentration. Except for the UV handlamp light source, the device shown in Fig. 6 was battery powered and could be easily designed as a portable instrument for bedside use.

DISCUSSION

The results shown for ANS-LDH and the polarization sensor represent only the first step toward development of a useful point-of-care lactate sensor. The beef heart LDH used in these experiments was only moderately stable at room temperature and had to be used within several days following removal from the ammonium sulfate solution.

This difficulty may be minimized by immobilization of the LDH into a matrix which often stabilizes proteins (30). However, we believe that several other steps should be taken prior to selection of the final protein sensor. Proteins from thermophilic organisms are known to display exceptional thermal stability (31, 32), with some proteins displaying activity above 100°C (33). Additionally, it would be desirable to obtain a larger spectral change, preferably with a change in lifetime or a useful spectral shift. Considerable information is available

about LDH and its conformational changes upon binding lactate (34, 35). For instance, binding of lactate results in motion of a surface peptide loop by about 10 Å to cover the active site. Also, LDH displays classic hinge motions upon substrate binding. Furthermore, lactate dehydrogenase has been isolated from thermophilic and mesophilic bacteria (36). This information, in combination with site-directed mutagenesis, should allow development of a stable lactate sensor protein displaying large and useful spectra changes. In turn, such a lactate sensor protein would allow development of a bedside lactate monitor.

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APPENDIX

Consider the apparatus shown in the top panel of Fig. 6. The sample (S) and reference (R) sides of the sensor are illuminated with a UV hand lamp. The emission from the reference passes through a vertically oriented polarizer, and the emission from the sample passes through a horizontally oriented polarizer. The emission from S and R is observed through an analyzer polarizer (AP) using a dual photocell.

The emissions passing through analyzer polarizer from the vertical and horizontal sides of the sensor are given by

$$I^V = I_{\parallel}^R \cos^2 \alpha \quad [A1]$$

$$I^H = I_{\perp}^S \sin^2 \alpha, \quad [A2]$$

where α is the angular displacement of the analyzer polarizer from the vertical position. The analyzer is rotated until the intensity from reference and sample are equal. For this condition one has

$$I_{\parallel}^R \cos^2 \alpha = I_{\perp}^S \sin^2 \alpha \quad [A3]$$

and

$$\tan^2 \alpha = \frac{I_{\parallel}^R}{I_{\perp}^S}. \quad [A4]$$

A change in the sample intensity induced by the analyte results in changes of the analyzer polarizer angle needed to equalize the intensities. We call this difference the compensation angle, α

$$\Delta \alpha = \alpha_0 - \alpha. \quad [A5]$$

In this expression α_0 refers to the rotation angle of analyzer polarizer (AP) needed to equalize the intensities in the absence of analyte and α is the angle of AP needed to equalize the reference and sample intensities in the presence of given analyte concentration (28, 29).

To obtain the maximum change in compensation angle, $\Delta\alpha$, one should consider the initial intensity ratio of reference to sample fluorescence intensities $k = \bar{I}^R/\bar{I}^S$, where $\bar{I}^R$ and $\bar{I}^S$ are the total intensities. Suppose the intensity of the sensing fluorophore decrease n times in response to analyte. Then the change in compensation angle is given by

$$\Delta\alpha = \text{atan}\sqrt{\frac{I^R}{I^S}} - \text{atan}\sqrt{\frac{nI^R}{I^S}}. \quad [\text{A6}]$$

Figure 6 shows simulations of the expected changes in compensation angle α as a function of initial ratio of the reference to sample fluorescence, k , for different values of n .

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FIG. 1.

Intrinsic fluorescence of LDH in the absence and presence of lactate. Increase of lactate concentration over 200 μM does not introduce further changes in fluorescence intensity.

FIG. 2.

Frequency-domain intensity decay of the intrinsic fluorescence of LDH in the absence and presence of lactate. The thin dashed line in the lower panel shows the decay in absence of lactate.

FIG. 3.

Emission spectra of ANS-labeled LDH in the presence of increasing concentrations of lactate. [LDH] = 3 μM . [ANS] = 4 μM . Increase of lactate concentration over 240 μM does not introduce further changes in fluorescence intensity.

FIG. 4.
Relative emission intensity of ANS-labeled LDH in the presence of increasing concentrations of lactate.

FIG. 5.

Frequency-domain intensity decay of ANS-labeled LDH in the absence and presence of lactate. The thin dashed line in the lower panel shows the decay in absence of lactate.

FIG. 6. Schematic of polarization sensing (top) and simulations of the expected changes in compensation angle for different values of n .

FIG. 7.
Polarization sensing of lactate bound on the emission intensity of ANS-labeled LDH.

TABLE 1

Multiexponential Intensity Decay of the Intrinsic Emission of LDH in the Absence and Presence of Lactate

[Lactate]	$\langle \tau \rangle$ (ns) ^a	$\bar{\tau}$ (ns) ^b	a_i	f_i	τ_i (ns)	χ^2_R
0.0	3.21	5.54	0.319	0.057	0.60	0.8 ^c
			0.399	0.312	2.60	
			0.282	0.631	7.47	
240 μ M	3.21	5.33	0.339	0.066	0.63	0.6
			0.363	0.280	2.48	
			0.298	0.653	7.05	

$$^a \langle \tau \rangle = \sum_j a_j \tau_j.$$

$$^b \bar{\tau} = \sum_i f_i \tau_i.$$

^cThe uncertainties in the phase and modulation were taken as $\delta\phi = 0.30$ and $\delta m = 0.007$, respectively.

TABLE 2

Multiexponential Intensity Decay of ANS-Labeled LDH in the Absence and Presence of Lactate

[Lactate]	$\langle \tau \rangle$ (ns) ^a	$\bar{\tau}$ (ns) ^b	a_i	f_i	τ_i (ns)	χ^2_R
0.0	9.73	14.8	0.217	0.017	0.78	0.8 ^c
			0.290	0.154	5.16	
			0.493	0.829	16.9	
240 μ M	9.01	14.4	0.265	0.022	0.77	0.7
			0.324	0.208	5.89	
			0.412	0.770	17.1	

$$^a \langle \tau \rangle = \sum_j a_j \tau_j.$$

$$^b \bar{\tau} = \sum_i f_i \tau_i.$$

^cThe uncertainties in the phase and modulation were taken as $\delta\phi = 0.30$ and δm , respectively.