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Single-molecule site-specific detection of protein phosphorylation with a nanopore

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Abstract

We demonstrate single-molecule site-specific detection of protein phosphorylation with protein nanopore technology. A model protein, thioredoxin, was phosphorylated at two adjacent sites. Analysis of the ionic current amplitude and noise, as the protein unfolds and moves through an α -hemolysin pore, enables the distinction between unphosphorylated, monophosphorylated and diphosphorylated variants. Nanopore proteomics based on these developments may prove valuable in basic science and the clinic.

The functional properties of most proteins are regulated by post-translational modifications of specific residues. Importantly, multi-site modifications can occur in different combinations, leading to different functional forms of a protein⁶. Phosphorylation at serine, threonine or tyrosine is the most frequent experimentally determined PTM¹. However, despite advances in high-resolution mass spectrometry of protein fragments, the determination of patterns of phosphorylation within individual protein molecules remains challenging. The occupancy and connectivity of phosphorylation sites is a problem ideally suited for single-molecule approaches. Engineered protein nanopores have been used for the stochastic detection of a wide variety of molecules in solution^{12, 13} and as an ultra-rapid, low-cost platform for single-molecule sequencing of DNA and RNA^{17, 18}. Further, proteins have been unfolded during forced translocation through the α -hemolysin (α HL) pore^{37, 38}, revealing distinct steps in the unfolding process³⁷. In the latter case, the model protein, thioredoxin (Trx), was tagged on a C-terminal cysteine with oligo(dC)₃₀. In an applied

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AUTHOR CONTRIBUTIONS

C.B.R. performed experiments, analyzed data and wrote the paper. D.R.-L. planned the research, performed experiments, analyzed data and wrote the paper. H.B. planned the research and wrote the paper.

COMPETING FINANCIAL INTERESTS

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potential, the DNA leader sequence threads into the α HL pore (step 1→2, Fig. 1) and exerts a force on the folded protein, which causes unfolding of a C-terminal domain (step 2→3). The remainder of the protein then unfolds spontaneously (step 3→4) and diffuses through the pore (step 4→1)³⁷. Here, we have examined a set of mutant Trx with phosphorylation sites for the catalytic subunit of protein kinase A (PKA) at several locations near the C terminus. By examination of changes in the ionic current when the C-terminal sequence moves into the pore, we have distinguished unphosphorylated, monophosphorylated and diphosphorylated states of the protein.

We first studied TrxS112^{-P}, a mutant derived from Trx V5 with a PKA phosphorylation site (RRAS) at the C terminus, where the underlined target serine is Ser-112 (Fig. 1). Trx V5 incorporates the mutations A22P, I23V, C32S, C35S and P68A, and carries a C-terminal cysteine for the attachment of a DNA leader³⁷. TrxS112^{-P}-oligo(dC)₃₀ was translocated into the α HL pore under an applied potential of +140 mV and produced the ionic current signature (Fig. 1 and Supplementary Fig. 1) described previously, which is characteristic of cotranslocational unfolding³⁷. We then phosphorylated TrxS112^{-P} at Ser-112 to give TrxS112^{+P} by overnight incubation with ATP and PKA. Phosphorylation was complete as determined by ESI LC-MS (Supplementary Fig. 2). Oligo(dC)₃₀ was then attached at the C terminus. TrxS112^{+P}-oligo(dC)₃₀ underwent cotranslocational unfolding by the same 4-step pathway as TrxS112^{-P}-oligo(dC)₃₀ and exhibited similar translocation kinetics (Supplementary Fig. 3). However, after phosphorylation, level 3 differed in mean residual current (I_{RES}) and noise (I_n) (Fig. 1). I_n is the standard deviation of a Gaussian fit to an all-points histogram of the ionic current. Detailed noise analysis revealed that, in general, phosphorylation produces a decrease in the low frequency spectral density and a small increase in the corner frequency (f_C , Supplementary Fig. 4). TrxS112^{-P}-oligo(dC)₃₀ gave $I_{RES}\% = 18.7 \pm 0.2\%$ of the open pore current, and $I_n = 6.0 \pm 0.1$ pA (100 events). With the same pore, TrxS112^{+P}-oligo(dC)₃₀ gave $I_{RES}\% = 20.9 \pm 0.2\%$, and $I_n = 5.4 \pm 0.2$ pA (100 events) (Fig. 1). We examined the voltage dependences of the ionic currents and found the largest differences between TrxS112^{-P} and TrxS112^{+P} in both $I_{RES}\%$ and I_n at +140 mV (Supplementary Fig. 5).

To affirm the ability of the α HL pore to distinguish phosphorylation at other locations, we made two additional mutants based on Trx V5: TrxS107^{-P}, with a phosphorylation site (RRNS) at position Ser-107 in the C-terminal α -helix of thioredoxin and TrxS95^{-P}, with a phosphorylation site (RRLS) at position Ser-95, in a loop that immediately precedes the C-terminal α -helix (Fig. 1). After coupling to oligo(dC)₃₀, all three non-phosphorylated proteins (TrxS95^{-P}, TrxS107^{-P} and TrxS112^{-P}) produced the characteristic 4-step signal (Supplementary Fig. 6). Nevertheless, the values of $I_{RES}\%$ and I_n in level 3 differed (Fig. 1), which we attribute to the exquisite ability of nanopores to distinguish between molecules located in the lumen of the pore^{39–41}. We examined each unphosphorylated and phosphorylated protein pair with the same α HL pore in order to avoid minor differences originating from pore-to-pore variation (Supplementary Table 1). TrxS107^{-P}-oligo(dC)₃₀, with the phosphorylation site in the C-terminal α -helix, gave $I_{RES}\% = 16.4 \pm 0.2\%$ and $I_n = 6.4 \pm 0.1$ pA (99 events) for level 3. We obtained almost complete phosphorylation of TrxS107^{-P} after 48 h as estimated by ESI LC-MS. After the attachment of oligo(dC)₃₀, a 4-step signal was obtained with $I_{RES}\% = 17.9 \pm 0.2\%$ and $I_n = 5.5 \pm 0.2$ pA for level 3 (100

events, Fig. 1). TrxS107^{-P}-oligo(dC)₃₀ and TrxS107^{+P}-oligo(dC)₃₀ could therefore readily be distinguished as two separate populations in 2D I_{RES}% versus I_n scatter plots displaying multiple translocation events (Fig. 1, Supplementary Fig. 4). Similarly, before phosphorylation, TrxS95^{-P}-oligo(dC)₃₀, with the phosphorylation site in the loop, displayed a level 3 with I_{RES}% = 22.5 ± 0.1% and I_n = 4.9 ± 0.1 pA (100 events). After treatment with PKA and ATP for 72 h, partial phosphorylation was obtained as judged by ESI LC-MS. Upon oligo(dC)₃₀ attachment and examination with the αHL pore, the phosphorylated and unphosphorylated forms could be distinguished. TrxS95^{+P}-oligo(dC)₃₀ gave a level 3 with I_{RES}% = 21.6 ± 0.3% and I_n = 3.8 ± 0.1 pA (150 events) (Fig. 1, Supplementary Fig. 4).

We next made TrxS107^{-P}/S112^{-P}, with two phosphorylation sites, one in the C-terminal α-helix (RRLS, Ser-107) and one in the C-terminal extension (RRAS, Ser-112), separated by an alanine residue (Fig. 1). Two control constructs were made containing single phosphorylation sites, by mutating Ser-107 of TrxS107^{-P}/S112^{-P} to Ala (TrxA107/S112^{-P}) and Ser-112 to Ala (TrxS107^{-P}/A112). All three constructs carried a C-terminal cysteine (Cys-113) for oligo(dC)₃₀ attachment. Again, these proteins showed the characteristic 4-step signal upon translocation through the αHL pore (Fig. 2). In all three cases, level 3 of the unphosphorylated constructs comprised two rapidly interconverting sub-states. The sub-state with the lowest conductance was used for comparison with the phosphorylated proteins, which show only the lower state, as judged by I_{RES}% values. The unphosphorylated proteins gave: TrxS107^{-P}/S112^{-P}-oligo(dC)₃₀ I_{RES}% = 13.0 ± 0.2%, I_n = 7.9 ± 0.4 pA (100 events); TrxA107/S112^{-P}-oligo(dC)₃₀ I_{RES}% = 12.3 ± 0.2%, I_n = 8.4 ± 0.6 pA (100 events); TrxS107^{-P}/A112-oligo(dC)₃₀ I_{RES}% = 13.2 ± 0.2%, I_n = 8.7 ± 0.6 pA (100 events) (Supplementary Figs. 7, 8). The detectable differences in I_{RES}% and I_n again illustrate the ability of protein nanopores to distinguish subtle variations in polypeptide structure (i.e. Ser→Ala). We next phosphorylated each construct with PKA. Complete phosphorylation of TrxA107/S112^{-P} took less than 2 h, whereas TrxS107^{-P}/A112 and TrxS107^{-P}/S112^{-P} required 44 h with replenishment of the reagents to attain almost complete phosphorylation (Supplementary Fig. 9). The phosphorylated constructs again showed distinctive I_{RES}% and I_n values in level 3 (Fig. 2): TrxS107^{+P}/S112^{+P}-oligo(dC)₃₀ I_{RES}% = 15.2 ± 0.2%, I_n = 5.7 ± 0.1 pA (98 events); TrxS107^{-P}/S112^{-P}-oligo(dC)₃₀ I_{RES}% = 12.6 ± 0.1%, I_n = 6.2 ± 0.2 pA (100 events); TrxA107/S112^{+P}-oligo(dC)₃₀ I_{RES}% = 13.8 ± 0.2%, I_n = 6.8 ± 0.2 pA (65 events); TrxS107^{+P}/A112-oligo(dC)₃₀, I_{RES}% = 14.6 ± 0.2%, I_n = 5.1 ± 0.2 pA (79 events) (Fig. 2 and Supplementary Figs. 4,10).

Based on the ability to distinguish monophosphorylation at Ser-107 and Ser-112, and phosphorylation of both sites, we examined the incomplete phosphorylation of TrxS107^{-P}/S112^{-P}. We monitored the time-course of phosphorylation by isoelectric focusing (IEF) (Fig. 2), which showed that after 2 h two populations of phosphorylated protein are present, one doubly phosphorylated and the other phosphorylated at just one site. IEF cannot distinguish phosphorylation at Ser-107 from phosphorylation at Ser-112. However, ESI LC-MS showed that TrxA107/S112^{-P} is almost completely phosphorylated after 2 h, whereas TrxS107^{-P}/A112 is not fully phosphorylated even after 48 h. Therefore, the two phosphorylated species derived from TrxS107^{-P}/S112^{-P} after 2 h are likely to be that with only Ser-112 phosphorylated and that with both sites phosphorylated. After 2 h of phosphorylation, a sample was tagged with oligo(dC)₃₀ and subjected to nanopore analysis,

which showed that the mixture contained two populations, one with $I_{\text{RES}}\% = 15.4 \pm 0.1\%$, $I_n = 5.7 \pm 0.1$ pA and the other with $I_{\text{RES}}\% = 14.3 \pm 0.1\%$, $I_n = 6.7 \pm 0.1$ pA (Fig. 2). Based on calibration of the nanopore (Supplementary Fig 11), these species correspond to the doubly phosphorylated species TrxS107^{+P}/S112^{+P} ($I_{\text{RES}}\% = 15.4 \pm 0.2\%$, $I_n = 5.7 \pm 0.1$ pA) and the protein phosphorylated on Ser-112, represented by TrxA107/S112^{+P}-oligo(dC)₃₀ ($I_{\text{RES}}\% = 14.0 \pm 0.3\%$, $I_n = 6.9 \pm 0.3$ pA). By contrast, TrxS107^{+P}/A112-oligo(dC)₃₀, gave $I_{\text{RES}}\% = 14.9 \pm 0.1\%$, $I_n = 5.2 \pm 0.1$ pA and TrxS107^{-P}/S112^{-P}-oligo(dC)₃₀ gave $I_{\text{RES}}\% = 13.0 \pm 0.1\%$, $I_n = 6.5 \pm 0.1$ pA). From the IEF band intensities, the sample was estimated to contain 39% doubly phosphorylated and 61% singly phosphorylated Trx. In accord with this, based on 202 single-molecule events, we found 67 molecules (33%) to be diphosphorylated and 123 molecules (61%) to be monophosphorylated at Ser112 with just one (0.5%) monophosphorylated at Ser107. Eleven events (5.5%) were unclassified.

Several improvements can be envisaged that would build upon our initial findings. For example, a future goal is to analyze more widely spaced phosphorylation sites. To achieve this goal, it may be necessary to pull a polypeptide continuously through a pore³⁸, rather than allow it to diffuse. Alternatively, the use of 'brakes', such as antibodies, could temporarily stop protein translocation, allowing different segments of the polypeptide chain to be examined. Another objective is to analyze modified proteins taken from cells. To this end, it will be necessary to enrich the protein target, and selectively modify the N or C terminus with a charged polymer so that the protein can be subjected to cotranslocational unfolding. Selective terminal modification can be achieved at specific residues^{42–43} or tags⁴⁴ by chemical or enzymatic means, or a charged sequence might be entirely genetically encoded⁴⁵. These approaches will be useful in laboratory experiments with transformed cells. For clinical samples, a more general terminal modification procedure will be required^{46, 47} to implement diagnostic "nanopore proteomics". To judge from the rapid development of nanopore sequencing^{17–31}, applications of nanopore proteomics could be available in as few as two or three years.

METHODS

Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Single-molecule nanopore detection of phosphorylation of a model substrate. **(a)** Current signature of the unfolding and translocation of TrxS112^{-P}-oligo(dC)₃₀ through an α HL pore showing the four characteristic levels: (1) open pore; (2) oligonucleotide leader threaded into the pore; (3) C terminus of the protein substrate in the pore; (4) unfolding of the remainder of the protein and diffusion through the pore. **(b)** Sequences of the C termini of Trx mutants used in this work. Phosphorylatable Ser residues, red; non-phosphorylatable Ala residues, black. **(c)** Model of TrxS112^{-P}: phosphorylatable Ser-112, red ball. **(d)** Current signature of TrxS112^{-P}-oligo(dC)₃₀. **(e)** Signature of TrxS112^{+P}-oligo(dC)₃₀. **(f)** Representative scatter plot of the residual current (I_{RES} %) and noise (I_n) in levels 3 of TrxS112^{-P}-oligo(dC)₃₀ and TrxS112^{+P}-oligo(dC)₃₀ and the associated histograms (200 events were recorded). **(g)** Model of TrxS107^{-P}, phosphorylatable Ser-107, red ball. **(h)** Current signature of TrxS107^{-P}-oligo(dC)₃₀. **(i)** Signature of TrxS107^{+P}-oligo(dC)₃₀. **(j)** Representative scatter plot of I_{RES} and I_n in levels 3 of TrxS107^{-P}-oligo(dC)₃₀ and TrxS107^{+P}-oligo(dC)₃₀ and the associated histograms (199 events). **(k)** Model of TrxS95^{-P}, phosphorylatable Ser-95, red ball. **(l)** Current signature of TrxS95^{-P}-oligo(dC)₃₀. **(m)** Signature of TrxS95^{+P}-oligo(dC)₃₀. **(n)** Representative scatter plot of I_{RES} and I_n in levels

3 of TrxS95^{-P}-oligo(dC)₃₀ and TrxS95^{+P}-oligo(dC)₃₀ and the associated histograms (250 events). All measurements were done at +140 mV. The experiments in (f), (j) and (n) were repeated three times.

Figure 2.

Single-molecule nanopore detection of four phosphorylation states. (a–f) Representative ionic current traces at +140 mV for TrxS107^{-P}/S112^{-P} (a), TrxS107^{+P}/S112^{+P} (b), TrxA107/S112^{-P} (c), TrxA107/S112^{+P} (d), TrxS107^{-P}/A112 (e) and TrxS107^{+P}/A112 (f). The Trx constructs were fused at the C terminus to oligo(dC)₃₀. (g) Representative scatter plot and histograms of the residual currents ($I_{RES}\%$) and noise (I_n) at +140 mV in level 3 (Fig. 1) for the four phosphorylation states in a, b, d and f. Only the low conductance substate is analyzed (see Supplementary Fig. 10). The same pore was used throughout and the cis compartment was perfused before the addition of each Trx variant. In total, 342 events were recorded, and a few events may be due to carry over because of incomplete perfusion. The ability of the same pore to distinguish the four constructs was verified twice. (h–i) Single-molecule nanopore detection of a sample containing a mixture of phosphorylation states. (h) Fraction of TrxS107^{-P}/S112^{-P} converted over time to the doubly phosphorylated TrxS107^{+P}/S112^{+P} as determined by IEF. (i) Representative scatter plot and histograms of $I_{RES}\%$ and I_n in level 3 after 2 h of phosphorylation, followed by conjugation to oligo(dC)₃₀ and α HL nanopore analysis at +140 mV. In total, 205 events recorded, of which three were discarded as too short (<30 ms) for analysis. The boxes delimit areas obtained as shown in Supplementary Figure 11. The ability to distinguish the components of the mixture was verified with a second pore.

Figure 3.