# Metal Ligand Affinity Pipettes and Bioreactive Alkaline Phosphatase Probes

TOOLS FOR CHARACTERIZATION OF PHOSPHORYLATED PROTEINS AND PEPTIDES*

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**An alkaline phosphatase-bioreactive probe, in which the enzyme is covalently bound to the mass spectrometry target, has been developed for studies of phosphoproteins. The bioreactive probe was used in combination with affinity capture and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to study hydrolysis of several phosphoproteins found in human saliva. Human salivary proteins were extracted from diluted human saliva with immobilized metal-affinity pipettes, which under defined conditions bound the phosphoproteins of interest preferentially over histatins. Phosphoproteins were eluted directly from the affinity pipettes to the bioreactive probe with diluted ammonium hydroxide, which provided conditions appropriate for hydrolysis by the alkaline phosphatase covalently bound to the probe surface. Results indicate the combination of metal-affinity pipette extraction, alkaline phosphatase-bioreactive probes, and matrix-assisted laser desorption/ionization mass spectrometry is an effective way to find and characterize phosphoproteins, known and unknown, in complex mixtures. Facile hydrolysis of human salivary phosphoproteins by the bioreactive probes was readily observed.** *Molecular & Cellular Proteomics 3:266–272, 2004.*

Posttranslational modification is an important aspect of expression of biologically active proteins. Primary translation products may undergo modifications, ranging from quite simple (N-terminal acetylation or C-terminal amidation) to more complex additions (glycosylation, phosphorylation, farnesylation) or proteolytic cleavages to generate the final active product. Developing the capabilities for determining the nature of the modifications and their positions within a protein sequence is a challenging problem that requires considerable time and effort when standard biochemical methods are used as tools. For example, radiolabeling and two-dimensional gel electrophoresis are often used to determine the extent and positions of protein phosphorylation. This combination of methods is time consuming and requires isotopic techniques with their attendant problems. Moreover, *in vivo* radiolabeling is not a generally accepted practice for studies with human subjects. Newer mass spectrometric methods are coming of age in addressing the general problem of posttranslational modifications, including protein phosphorylation (for reviews see Refs. 1–4). Studies of phosphorylation with mass spectrometry-based methods often rely on two-dimensional gel electrophoresis, capillary electrophoresis, high-performance liquid chromatography (HPLC),[1] immunocapture or chemical means to concentrate proteins and peptides (5–13) with isotope labeling as a common means for detection of phosphorylated proteins and peptides for subsequent mass spectrometric analyses. Stensballe and coworkers (14) recently used Fe(III) affinity chromatography to capture phosphoproteins, followed by digestion with added alkaline phosphatase to characterize peptides from phosphoproteins. Electrophoretic separation of $^{32}$P-labeled proteins and subsequent *in situ* digestion of dissected sections with proteases were used, in conjunction with added on-target alkaline phosphatase digestion and subsequent mass spectrometry, to identify phosphopeptides from more than 20 proteins in extracts from HeLa 299 cells (15).

The work described in this article uses directed approaches to retrieve phosphorylated proteins from salivary extracts and to partially characterize these proteins with respect to the extent of phosphorylation. This combined method (16–19), illustrated in Fig. 1, relies on the dynamic interplay between affinity pipettes and a bioreactive probe array to which alkaline phosphatase has been covalently attached and stabilized. Proteins, loaded on the metal chelate-conjugated surfaces of microcolumns integrated within the pipette tips (affinity pipettes), are directly released to the alkaline phosphatase-bioreactive probes on the array and are analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). The results obtained by hydrolysis of phosphoproteins, extracted from human saliva

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[1] The abbreviations used are: HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; PNP, *p*-nitrophenyl phosphate; TFA, trifluoroacetic acid; EDTA-DA, ethylendiaminotetraacetic acid dianhydride; DTPA-DA, diethyltriaminepentaacetic acid dianhydride; DSP, dithiobis[succinimidylpropionate]; DMA, dimethyladipimidate; ODS, octadecane-18-thiol; Tris, trishydroxymethyl aminomethane; ACN, acetonitrile; NTA, nitrilotriacetic acid.



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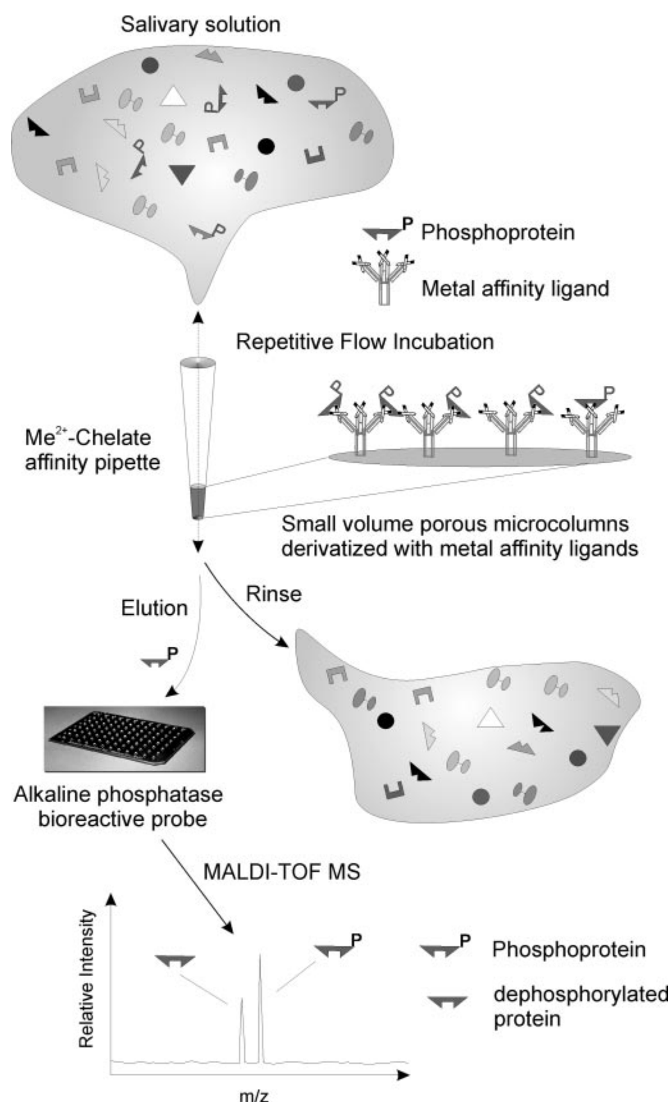


FIG. 1. **Flow diagram of affinity pipette capture/bioreactive probe digestion/mass spectrometry process for human salivary phosphoproteins.** Proteins were extracted and concentrated by repetitive flow through metal chelate affinity pipettes, then with dilute ammonium hydroxide. The enzymatic hydrolysis reaction was carried out at ambient temperature in a humidity chamber.

with affinity pipettes, clearly show mass-resolved analytes consistent with the removal of one or more phosphate residues. This direct and simple method is rapid, sensitive, and amenable to automation, allowing it to be used for screening large numbers of samples.

## EXPERIMENTAL PROCEDURES

*Chemicals and Reagents*—Dithiobis[succinimidylpropionate] (DSP), dimethyladipimidate (DMA), (Pierce Chemical Co., Rochester, NY); octadecane-18-thiol (ODS; Aldrich, Milwaukee, WI), trishydroxymethyl aminomethane (Tris), $\alpha$-cyano-4-hydroxy cinnamic acid, calf intestine alkaline phosphatase, *p*-nitrophenyl phosphate (PNP; Sigma Chemical Co., St. Louis, MO); ethylendiaminotetraacetic acid dianhydride (EDTA-DA); diethyltriaminepentaacetic acid dianhydride (DTPA-DA) and *N*,*N*-(Bis(carboxymethyl)-L-lysine hydrate (Fluka, Buchs, Switzerland); HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20). Inorganic salts and organic solvents were reagent-grade materials from reputable suppliers. High-purity water, used for making all solutions, and trifluoroacetic acid (TFA) were obtained from American Bioanalytical (Shelton, CT).

*Coupling of Alkaline Phosphatase to Mass Spectrometry Plates*—The targets of a gold-surfaced mass spectrometry plate were activated with DSP using a procedure similar to that described by Nelson (16). Alkaline phosphatase (0.3 mg/ml) was prepared in 0.1 M sodium borate containing 1 mM $MgCl_2$ and 25 $\mu$M $ZnSO_4$, adjusted to pH 9.5 with NaOH. Activated targets were incubated with 2 $\mu$l of alkaline phosphatase solution for 1 h at room temperature in a humidified chamber. Two microliters of DMA 10 mg/ml, dissolved in the same buffer used to dissolve alkaline phosphatase, was then added, and incubation was continued for 1 h to achieve cross-linking of subunits (20). The plate was rinsed with 0.1 M Tris buffer containing 1 mM $MgCl_2$ and 25 $\mu$M $ZnSO_4$ at pH 9.5, then incubated with storage buffer (0.1 M Tris, 1 mM $MgCl_2$, 25 $\mu$M $ZnSO_4$, pH 8.1) for 30 min to remove any unreacted succinimide residues from the probe surface. The plate was allowed the to dry in the presence of storage buffer and stored at room temperature in a closed chamber.

*Qualitative Assessment of Bioreactive Probe Alkaline Phosphatase Activity*—To ensure active alkaline phosphatase-bioreactive probes, qualitative experiments were performed with PNP, a commonly used alkaline phosphatase organic substrate (21). Activity of alkaline phosphatase solutions was tested qualitatively by adding 1 $\mu$l of diluted stock alkaline phosphatase (0.03 mg/ml) to 100 $\mu$l of PNP dissolved in 0.05 M sodium borate, 1 mM $MgCl_2$, 25 $\mu$M $ZnSO_4$ at pH 9.5, which led to rapid development of yellow color from the generation of *p*-nitrophenol. Color was not observed when an equal volume of buffer was added to an equivalent amount of this substrate as a negative control. Selected bioreactive probes on the mass spectrometry plate were tested for alkaline phosphatase activity by addition of 5 $\mu$l of PNP substrate solution. After incubation for 5 min, the incubation solution was transferred to a white surface to observe the presence of the yellow color produced by the hydrolysis of PNP. Activity of the alkaline phosphatase-bioreactive probes was readily apparent from the yellow color produced by the incubation with 5 $\mu$l of PNP substrate. Color was not observed when PNP was incubated with alkaline phosphatase-bioreactive probes to which 1 $\mu$l of 0.2% TFA was added prior to addition of PNP substrate or to targets that lacked alkaline phosphatase.

*Preparation of Immobilized Metal Ion Affinity Pipettes*—Metal chelators incorporated into porous glass microcolumns are useful agents for binding metal ions, which, in turn, can be used to capture biomolecules from solution. Amine or polyamine microcolumns, prepared in a manner similar to that described by Tubbs *et al.* (17), were overlaid in 0.1 M phosphate at pH 7 and evacuated using a rotary evaporator. Primary amine surfaces were then directly coupled to bifunctional chelating agents, such as EDTA-DA or DTPA-DA at 20 mg/ml, generating chelate surfaces (Tris(carboxymethyl)ethylenediamine and EDTA, respectively) that are able to form metal coordination complexes. Quadradentate nitriloacetic acid (NTA) surfaces were prepared in a manner similar to that described by Huchuli *et al.* (8).

Chelator microcolumns, integrated into 200-$\mu$l pipette tips, were charged with $Ni^{2+}$ or $Co^{2+}$ with a Beckman Multimek 96 robotic system (Beckman Coulter, Fullerton, CA). The robot sequence was 0.12 M HCl (50 repetitions of 50 $\mu$l), HBS-P buffer (50 repetitions of 50 $\mu$l), water (50 repetitions of 50 $\mu$l), 0.5 M metal ion (100 repetitions of 50 $\mu$l), water (50 repetitions of 50 $\mu$l). The affinity pipettes were then used to efficiently extract and concentrate biomolecules.

*Procedure for Loading Affinity Pipettes with Human Salivary Proteins*—After rinsing the oral cavity with water, salivary samples were expelled directly into a 1.5-ml centrifuge tube and subjected to cen-

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trifugation for 7 min at 13,000 rpm in a VSCM-13 centrifuge from Shelton Scientific (Shelton, CT). An aliquot of the salivary solution was diluted 25-fold with HBS-P buffer or with 2.5 mM sodium acetate buffer. The diluted saliva was immediately used as a source of protein for extraction by the immobilized metal affinity pipettes described earlier. Affinity pipettes, attached to a motorized multichannel pipetter set at 150 $\mu$l, were rinsed five times with high-purity water, then loaded with protein by repetitive cycling of diluted saliva sample (50–100×) through the metal chelate affinity pipette. Affinity pipettes were then rinsed sequentially with 150 $\mu$l of water (5×), 20% acetonitrile in water (5×), and water (5×). These sequential rinse steps were patterned after procedures routinely employed in this laboratory to reduce nonspecific binding to affinity pipettes.

*Phosphate Cleavage from Human Salivary Proteins with Alkaline Phosphatase-bioreactive Probes*—The stored alkaline phosphatase-bioreactive probes were prepared for use by washing the plate with constant agitation in storage buffer. After the third 5-min wash, residual buffer was removed by lightly blotting the surface with a Kimwipe. The protein-loaded affinity pipettes were rinsed with 20 $\mu$l of 0.005% TFA (2×), followed by 5 $\mu$l of water (5×), prior to eluting proteins to the alkaline phosphatase-bioreactive probes. Bound proteins were then eluted from the metal ligand affinity pipettes by drawing 9 $\mu$l of ammonium hydroxide (25–100 mM) into the affinity pipette with a 20-$\mu$l Pipetman (Gilson, Middleton, WI) and cycling the ammonium hydroxide through the affinity pipette 50 times, then dispensing the eluate into a 0.65-ml centrifuge tube. Two microliters of the eluate was deposited on individual alkaline phosphatase-bioreactive probes. The array was then incubated in a humidified chamber at room temperature. Hydrolysis was terminated at designated times by addition of 3 $\mu$l of matrix ($\alpha$-cyano-4-hydroxy cinnamic acid in 0.6% TFA, 33% acetonitrile (ACN)) to each probe. Zero-time samples were obtained by adding 1 $\mu$l of 0.2% TFA to the probe prior to deposition of the eluate, and 3 $\mu$l matrix was added immediately after the protein eluate. The array was allowed to dry at room temperature, then subjected to MALDI-TOF MS. The rinses with 0.005% TFA were eliminated from the washing procedure when proteins were bound to NTA surfaces from saliva diluted in 2.5 mM sodium acetate. Proteins were eluted from these affinity pipettes directly with 100 mM ammonium hydroxide.

*Mass Spectral Analyses*—Mass spectrometry was carried out with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker, Billerica, MA). For direct analyses of human salivary proteins, a 96-target (8 × 12) sample stage was used, whereas bioreactive alkaline phosphatase probe analyses were done on 10 target discs using a sample stage with a 12-disc format. Linear delayed-extraction mode was employed with a 2.25-kV draw-out pulse (400 ns) with full accelerating potential of 19.5 kV. Cytochrome *c* was used as a protein calibration standard. Two hundred laser shots were summed and saved as individual spectra.

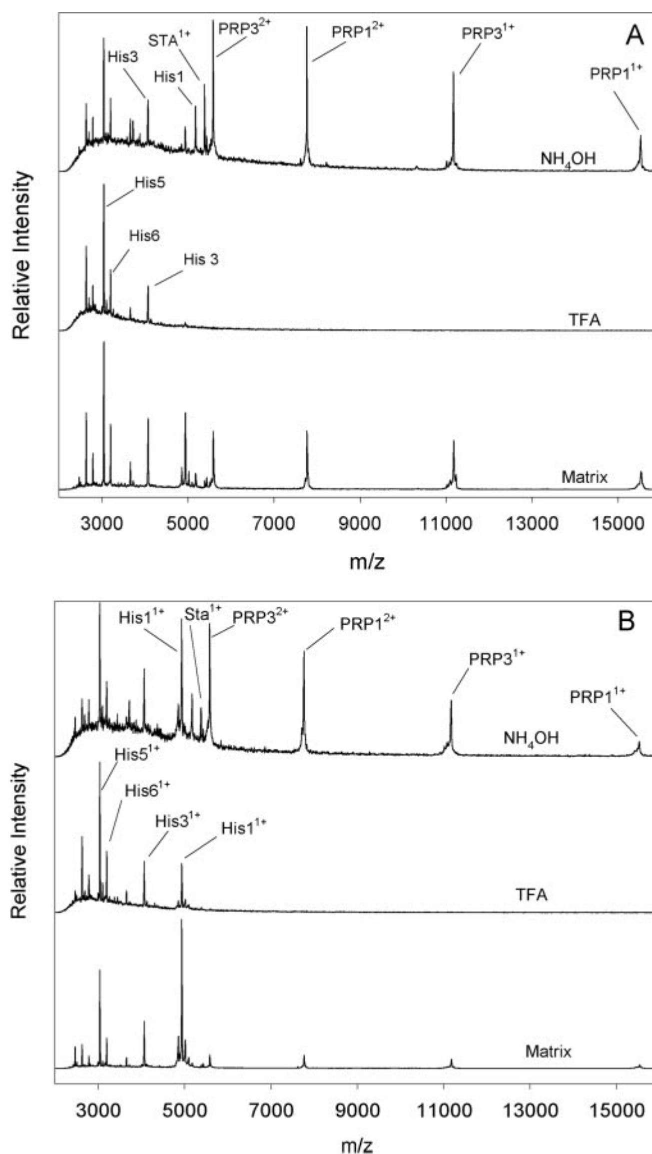


FIG. 2. **Representative mass spectra of proteins eluted from metal chelate affinity pipettes.** Proteins were extracted and concentrated from HBS-P-buffered saliva by repetitive flow through porous glass TED metal ion affinity pipettes. Proteins were eluted from three affinity pipettes directly to a 96-target mass spectrometer plate, using elution conditions described below. Mass spectra were generated in linear delayed-extraction mode. Each trace represents data normalized to a 0.0 to 1.0 scale prior to incorporation into the figure. *A*, TED-$Ni^{2+}$ surface. *Bottom*, 5 $\mu$l of matrix; *middle*, 5 $\mu$l of 0.005% TFA; *top*, 5 $\mu$l of 50 mM $NH_4OH$ after washing with 0.005% TFA. *B*, TED-$Co^{2+}$ surface. *Bottom*, 5 $\mu$l of matrix; *middle*, 5 $\mu$l of 0.005% TFA; *top*, 5 $\mu$l of 50 mM $NH_4OH$ after washing with 0.005% TFA. Readily identified proteins: PRP1 (15,537.7); PRP3 (11,179.7); statherin (5,388.1); histatin 1 (4,936.0); histatin 3 (4,066.1); histatin 6 (3,194.9); histatin 5 (3,039.4).

## RESULTS

Fig. 2 shows representative MALDI-TOF spectra of human salivary proteins eluted from TED-$Ni^{2+}$ and TED-$Co^{2+}$ immobilized metal ion affinity pipettes. The bottom traces in Fig. 2*A* (TED-$Ni^{2+}$) and Fig. 2*B* (TED-$Co^{2+}$) were obtained by direct elution with matrix. The middle trace in each case was obtained from solution eluted with 0.005% TFA, and the upper traces were derived from subsequent elution with 50 mM ammonium hydroxide. Table I lists some known phosphorylated and nonphosphorylated human salivary proteins and their molecular masses, derived from information presented in the Swiss-Pro/TRMBL database of the EXPASY web site. The abbreviations for the acidic proline-rich human salivary proteins used in this manuscript are based on sequence information presented by Hay *et al.* (22). A number of peaks in each trace of Fig. 2 correspond to known phosphorylated

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TABLE I
*Known metal-binding salivary proteins*

Masses were derived from cleavage of precursors, based on cleavage sites information presented in the Swiss-Prot and TrEMBL data base on the EXPASY website.

Note: These studies were repeated approximately 25-times over a 6-month period of time. All studies produced results comparable to those shown in this table. Mass variance (calculated − observed) was generally less than 0.1%, which is consistent with day-to-day variations associated with MALDI-TOF MS analysis using external calibration.

| Protein | Swiss-Prot accession no. | Residues | Mass | Mass + 1P | Mass + 2P |
|---|---|---|---|---|---|
| PRP C $HUMAN_{calc}$ | P02810 | 1–166 | 17,017 | | |
| PRP $C_{obs}$ | | | Not observed | | |
| PRP1/$2_{calc}$[a] | | 17–166 | 15,372.3 | 15,452.3 | 15,532.3 |
| PRP1/$2_{obs}$ | | | 15,372.7 | 15,452.4 | 15,532.6 |
| PRP3/$4_{calc}$[b] | | 17–122 | 11,019.5 | 11,099.5 | 11,179.5 |
| PRP3/$4_{obs}$ | | | 11,016.0 | 11,095.8 | 11,176.5 |
| Peptide P–$C_{calc}$ | | 123–166 | 4370.8 | | |
| Peptide P–$C_{obs}$ | | | Not observed | | |
| STAT_$HUMAN_{calc}$ | P2808 | 1–62 | 7304.5 | 7384.5 | 7464.5 |
| STAT_$HUMAN_{obs}$ | | | Not observed | | |
| $Statherin_{calc}$ | | 20–62 | 5219.7 | 5299.7 | 5379.7 |
| $Statherin_{obs}$ | | | | 5307.8 | 5388.1 |
| HIS1_$HUMAN_{calc}$ | P15515 | 1–57 | 6962.9 | | |
| HIS1_$HUMAN_{obs}$ | | | Not observed | | |
| Histatin $1_{calc}$ | | 20–57 | 4848.2 | 4928.2 | |
| Histatin $1_{obs}$ | | | 4856.2 | 4936.0 | |
| HIS3_$HUMAN_{calc}$ | P15516 | 1–51 | 6149.1 | | |
| HIS3_$HUMAN_{obs}$ | | | Not observed | | |
| Histatin $3_{calc}$ | | 20–51 | 4062.4 | | |
| Histatin $3_{obs}$ | | | 4066.1 | | |
| Histatin $5_{calc}$ | | 20–43 | 3036.3 | | |
| Histatin $5_{obs}$ | | | 3036.1 | | |
| Histatin $6_{calc}$ | | 20–44 | 3192.5 | | |
| Histatin $6_{obs}$ | | | 3192.9 | | |

[a] PRP1 represents three positional isomers, namely PRP-1, PRP-2, and PIF-s, using the nomenclature of Hay *et al.* (22).
[b] PRP3 represents three positional isomers, namely PRP-3, PRP-4, and PIF-f, using the nomenclature of Hay *et al.* (22).

salivary proteins, namely PRP1 (*m/z* 15,532.3) and PRP3 (*m/z* 11,179.5), derived from proline-rich precursor protein (accession no. P02810). Other known phosphorylated proteins apparent in the spectra include statherin (*m/z* 5,379.7), derived from STAT_HUMAN (accession no. P02808), and histatin 1 (m/z 4,928.2), derived from its precursor (accession no. P15515). A number of peaks in the lower-molecular-mass region of the spectra correlate with molecular masses of other histatins, derived from precursor proteins His1_Human (accession no. P15515) and His3_Human (accession no. P15516). The most intense of these peaks correspond to histatins 1, 3, 5, and 6 (see Table I for mass data). Extraction from acetate buffer at pH 5 with NTA affinity pipettes eliminated binding of most of the histatins and was used to reduce complexity of the spectra for the bioreactive probe phosphoprotein digestion experiments shown in Fig. 3.

Digestions of salivary proteins, eluted from NTA-$Ni^{2+}$and NTA-$Co^{2+}$ affinity pipettes, with alkaline phosphatase-coupled mass spectrometry-bioreactive probes are illustrated in Fig. 3. Reaction times (bottom to top) are 0, 10, and 20 min, respectively. Mass shifts of 80 amu or multiples thereof were observed for the known phosphorylated proteins from human saliva (masses are presented in Table I). The rates of hydrolysis of the different phosphoproteins extracted from human saliva were quite varied. Hydrolysis of PRP1, for example, is more extensive than PRP3 at each incubation time, as is apparent from examination of both the singly charged and the doubly charged forms of the proteins, even though both proteins are derived from the same phosphorylated precursor protein. A number of unknown phosphoproteins were observed at much lower intensities than the well-established human salivary phosphoproteins (labeled X-1 to X-4) in Fig. 3*A*. Fig. 3*C* shows two expanded regions of the spectra to more clearly illustrate the hydrolysis of three of the unknown proteins.

## DISCUSSION

The development of affinity pipettes and alkaline phosphatase-bioreactive probes used in this study for characterization of phosphoproteins was based on earlier studies (16–19) with antibodies and proteases. Studies of alkaline phosphatase by McCracken and Meighen (23) indicated the monomeric subunit of dimeric alkaline phosphatase does not have significant enzymatic activity. Washing and incubation steps in our early

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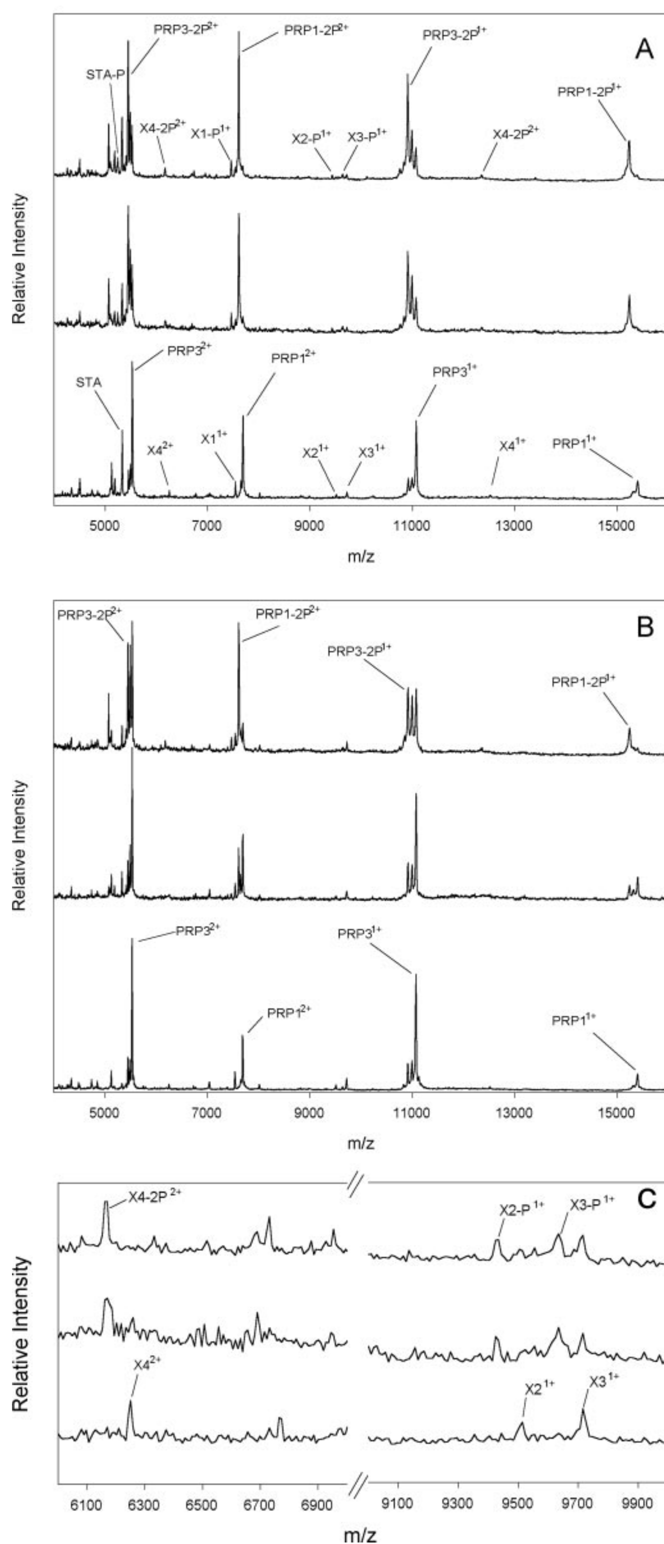


FIG. 3. **Alkaline phosphatase digestion of extracted salivary proteins by bioreactive probes.** Proteins were extracted with NTA metal ion affinity pipettes, and bound phosphoproteins were eluted with 9 $\mu$l of 100 mM ammonium hydroxide. Two microliters of the eluate was transferred to each bioreactive probe, and at selected incubation times enzymatic reactions were terminated by addition of matrix. MALDI-TOF MS was performed as described in Fig. 2. Each trace represents data normalized to a 0.0 to 1.0 scale prior to incorporation into the figure. *A*, NTA-$Ni^{2+}$ surface. *Bottom*, $t = 0$ min; *middle*, $t = 10$ min; *top*, $t = 20$ min. *B*, NTA-$Co^{2+}$ surface. *Bottom*, $t = 0$ min; *middle*, $t = 10$ min; *top*, $t = 20$ min. Identifiable phosphoproteins: PRP1 and hydrolysis products (*m/z*: 15,532.6, 15,452.4, 15,372.5; $\Delta m$ = 160.1 amu), PRP3 and hydrolysis products (*m/z*: 11,176.6, 11,095.8, 11,016.0; $\Delta m$ = 160.6 amu), statherin and hydrolysis product (*m/z*: 5,388.1, 5,307.8; $\Delta m$ = 80.3 amu). Four unknown phosphoproteins and generated hydrolysis products were observed. $X1^{1+}$ (*m/z*: 7,620.1, 7,540.3; $\Delta m$ = 79.8 amu), $X2^{1+}$ (*m/z*: 9,608.0, 9,527.9; $\Delta m$ = 80.1 amu), $X3^{1+}$ (*m/z*: 9,815.9, 9,735.7; $\Delta m$ = 80.2 amu), and $X4^{1+}$ (*m/z*: 12,636.6, 12,475.8; $\Delta m$ = 160.8 amu). *C*, An expanded view of two regions of the spectra in Fig 3*A* showing several low-level unidentified phosphoproteins and their hydrolysis products, namely $X2^{1+}$, $X3^{1+}$, and $X4^{2+}$ (*m/z*: 6,318.3, 6,238.2 amu).

studies, before inclusion of cross-linking, may have been responsible for loss of substantial enzymatic activity after storage. Washing steps used after storage to prepare the array for use could have led to dissociation of dimeric enzyme, leaving mostly monomeric alkaline phosphatase covalently attached to the probes. This could well account for the low activity observed. Cross-linking the bound alkaline phosphatase with a homobifunctional cross-linker (20), DMA in this case, led much higher levels of alkaline phosphatase activities on the probes. Similar results were obtained when dimethyl suberimidate and dimethyl pimelimidate were used as cross-linking agents. Bioreactive probe alkaline phosphatase activity has been observed after room temperature storage for more than 2 months, evidenced by hydrolysis of PNP and phosphorylated proteins from human saliva when chemical cross-linking was part of the preparation process. These data support the supposition that cross-linking preserves the enzymatic activity of the covalently bound alkaline phosphatase, most likely by preventing the dissociation of the active dimeric form of the enzyme (23). Final storage conditions were established empirically, and based on the results presented, alkaline phosphatase-bioreactive probes can be stored at room temperature for extended periods of time and still retain the ability to hydrolyze phosphorylated proteins.

The data presented in this manuscript, obtained with $Ni^{2+}$ and $Co^{2+}$ ligand columns, are part of a broader investigation that includes other ligands (EDTA, DPTA) and metal ions ($Ga^{3+}$, $Fe^{3+}$, $Cu^{2+}$). Based on our preliminary studies, $Ni^{2+}$ and $Co^{2+}$ affinity pipettes were selected for extraction of human saliva for these alkaline phosphatase-bioreactive probe studies. Ease of elution of human phosphoproteins with ammonium hydroxide, providing conditions that were compatible with enzymatically active bioreactive probes, was a primary consideration. The results clearly demonstrate the utility of the combination of affinity pipette extraction/concentration coupled to an alkaline phosphatase-bioreactive mass spectrometry probe and subsequent MALDI-TOF MS analysis for studies of human salivary phosphoproteins. The devices

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provided a sensitive means for rapid identification and evaluation of the level of phosphorylation of some phosphoproteins. The results obtained by affinity extraction of proteins from diluted human saliva demonstrate the potential for selective concentration of salivary phosphoproteins. Direct elution with ammonium hydroxide (25–100 mM) provided conditions under which alkaline phosphatase activity of the bioreactive probe was retained. Stopping the enzymatic reaction by addition of matrix gave conditions amenable to direct detection of proteins by MALDI-TOF MS. The data presented in Figs. 2 and 3, when compared with calculated values (see Table I) for known human salivary phosphoproteins, clearly show extraction of phosphoproteins by the affinity pipettes. When eluates from the affinity pipettes are incubated with alkaline phosphatase-bioreactive probes, sequential mass changes of 80 amu that correspond to loss of phosphoryl residues from PRP1, PRP3, statherin, histatin 1, and four unknown phosphoproteins are readily apparent.

Extraction of proteins from diluted human saliva was highly dependent on a number of factors, such as pH, chelator, and metal ion. The data in Fig. 2, *A* and *B* (*bottom traces*) indicate the TED-metal ion surfaces not only bind the phosphoproteins of interest, but also bind lower-molecular-mass nonphosphorylated proteins, including a number of histatins. Some of the low-molecular-mass substances dominate the intensity scale of the spectra. The rinse steps with 0.005% TFA effectively eluted most of these proteins without removing much phosphoprotein, thereby simplifying the resulting spectra. The effect of pH on extraction is quite substantial, because extraction from acetate buffer at pH 5.0 dramatically reduced the amounts of bound low-molecular-mass substances in the eluates. Detection of some low-intensity phosphoproteins was abetted by reducing levels of the dominant peaks in the lower mass range. The power of the combined affinity pipette/bioreactive probe technology is demonstrated by the detection of four previously unreported phosphoproteins in human saliva. Fig. 3*C* clearly shows the 80-amu shift for the two singly charged forms at *m/z* 9,608.0 and *m/z* 9,815.9. The 80-amu shift from *m/z* 6,318.3 to 6,238.2 represents the doubly charged species of the parent *m/z* 12,636.6. Whether these newly discovered salivary phosphoproteins are degradation products of the major species present in human saliva is not known at this time. Each of the four unknown phosphoproteins was mass matched against the known sequence of PRP1. In each instance, at least one peptide was found that included one or both of the known phosphate binding sites (positions 8 and 22). Thus, it seems quite possible that each of the unknown phosphoproteins arose by *in vivo* cleavage of PRP1 or its precursor, although in-source decay (gas-phase cleavage) cannot be ruled out. Further studies of metal ligand-extracted proteins from saliva in conjunction with tandem mass spectrometry are needed to definitively establish identity of the unknown phosphoproteins and any precursor-product relationships.

In summary, a method that combines metal chelate affinity pipette capture, bioreactive probe hydrolysis, and MALDI-TOF MS has been developed for studies of phosphoproteins. By merging these techniques with specific proteolytic digestions of the proteins, it should be possible to generate peptides, which if subjected to collision-induced dissociation, could provide data pertaining to sequences of the phosphorylated peptides. Furthermore, hydrolysis of protease digests on alkaline phosphatase-bioreactive probes will generate valuable information about the phosphorylation levels of the peptide fragments. Extension of this methodology to the study of protein phosphorylation/dephosphorylation cascades that serve as important control mechanisms for many biological processes is a reasonable expectation.

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REFERENCES

1. Mann, M., Ong, S.-E., Grønborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of phosphorylation using mass spectrometry: Deciphering the phosphoproteome. *Trends Biotechnol.* **20,** 261–268
2. McLachlin, D. T., and Chait, B. T. (2001) Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* **5,** 591–602
3. Zhang, X., Herring, C. J., and Qin, J. (2000) Identification of *in-vitro* phosphorylation sites with mass spectrometry, in *Mass Spectrometry in Biology and Medicine* (Burlingame, A. L., Carr, S. A., and Baldwin, M. A., eds.) pp. 309–328, Humana Press, Totowa, NJ
4. Dass, C. (1998) Analysis of phosphorylated proteins by mass spectrometry, in *Mass Spectrometry of Biological Materials* (Larsen, B. S., and McEwen, C. N., eds.) pp. 247–279, Marcel Dekker, New York
5. Marcus, K., Immler, D., Sternberger, J., and Meyer, H. E. (2000) Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. *Electrophoresis* **21,** 2662–2636
6. Conrad, T. P. Issaq, H. J., and Veenstra, T. D. (2002) New tools for quantitative phosphoproteome analysis. *Biochem. Biophys. Res. Commun.* **290,** 885–890
7. Cao, P., and Stults, J. T. (2000) Mapping the phosphorylation sites of proteins using on-line immobilized metal affinity chromatography/capillary electrophoresis/electrospray ionization multipole stage tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **14,** 1600–1606
8. Hochuli, E., Dobeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine residues. *J. Chromatogr.* **411,** 177–184
9. Graham, M. E., Dickson, P. W., Dunkley, P. R., and von Nagy-Felsobuki, E. I. (2000) Determination of phosphorylation levels of tyrosine hydroxylase by electrospray mass spectrometry. *Anal. Biochem.* **281,** 98–104
10. Gingras, A-C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burley, S. K., Polakiewicz, R. D., Wyslouch-Cieszynska, A., Aebersold, R. and. Sonenberg, N. (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* **15,** 2852–2864
11. Oda, Y., Nagasu T., and Chait, B. T. (2001) Enrichment analysis of phos-

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phorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **19,** 379–382

12. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White F. M. (2001) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae. Nat. Biotechnol.* **20,** 301–305
13. Zhou, H., Watts, J. D., and Aebersold, R. (2001) A systematic approach to analysis of protein phosphorylation. *Nat. Biotechnol.* **19,** 375–378
14. Stensballe, A., Andersen, S., and Jensen, O. N. (2001) Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **1,** 207–222
15. Larsen, M. R., Sørensen, G. L., Fey, S. J., Larsen, P. M., and Roepstorff, P. (2001) Phospho-proteomics: Evaluation of the use of enzymatic dephosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* **1,** 223–238
16. Nelson, R. W. (1997) The use of bioreactive probes in protein characterization. *Mass Spectrom. Rev.* **16,** 353–376
17. Tubbs, K. A., Nedelkov, D., and Nelson, R. W. (2001) Detection and quantification of $\beta$-2-microglobulin using mass spectrometric immunoassay. *Anal. Biochem.* **289,** 26–35
18. Kiernan, U. A., Tubbs, K. A., Gruber, K., Nedelkov, D., Niederkofler, E. E., Williams, P., and Nelson, R. W. (2002) High-throughput protein characterization using mass spectrometric immunoassay. *Anal. Biochem.* **301,** 49–56
19. Niederkofler, E. E., Tubbs, K. A., Gruber, K., Nedelkov, D., Kiernan, U. A., Williams, P., and Nelson, R. W. (2001) Determination of $\beta$-2 microglobulin levels in plasma using a high-throughput mass spectrometric immunoassay system. *Anal. Chem.* **73,** 3294–3299
20. Davies, G. E., and Stark, G. R. (1971) Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci., U. S. A.* **66,** 651–656
21. Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962) Alkaline phosphatase of *Escherichia coli*: A zinc metalloenzyme. *Biochemistry* **1,** 373–378
22. Hay, D. I., Bennick, A., Schlesinger, D. H., Minaguchi, K., Madapallimattam, G., and Schluckebier, S. K. (1988) The primary structures of six human salivary acidic proline-rich proteins (PRP-1, PRP-2, PRP-3, PRP-4, PIF-s and PIF-f). *Biochem. J.* **255,** 15–22
23. McCracken, S., and Meighen, E. (1987) Immobilized subunits of alkaline phosphatase. *Methods Enzymol.* **135,** 492–501

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