Analytical Biochemistry 397 (2010) 139–143

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Use of a semisynthetic epitope to probe histidine kinase activity and regulation

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ARTICLE INFO

Article history:
Received 28 August 2009
Received in revised form 5 October 2009
Accepted 6 October 2009
Available online 9 October 2009

Keywords:
Histidine kinase
ATPγS
Thiophosphate
Semisynthetic epitope
para-Nitrobenzylmesylate
Thiophosphohistidine
Thiophosphoaspartic acid

ABSTRACT

Histidine–aspartic acid phosphotransfer pathways are central components of prokaryotic signal transduction pathways and are also found in many eukaryotes. Tools to study histidine kinases, however, are currently quite limited. In this article, we present a new tool to study histidine–aspartic acid phosphotransfer pathways. We show that many histidine kinases will accept ATPγS as a substrate to form a stable thiophosphohistidine even when they do not form stable phosphohistidines using the natural substrate ATP. An antibody that has previously been used to detect thiophosphorylated serine, threonine, and tyrosine residues is shown to recognize thiophosphohistidine and thiophosphoaspartic acid residues. Histidine kinase autothiophosphorylation is regulated by other protein sensor domains in the same way as autophosphorylation, and thiophosphate is transferred to downstream aspartic acid containing response regulators.

Histidine kinases are important components of bacterial, fungal, and plant signal transduction pathways [1]. In response to environmental signals, the rates of histidine kinase autophosphorylation or phosphatase activity change, leading to an altered phosphorylation state of downstream response regulators. These changes lead to regulation of fundamental cellular processes such as flagellar rotation and cell growth state [1–3]. Currently, there are a limited number of tools available to study the activity of histidine kinases, and all use the natural substrate ATP and rely on the ability of the histidine kinase to form a stable phosphohistidine or to transfer the phosphoryl group to a downstream response regulator that is isolable [4–6]. The characterization of histidine kinases remains challenging due largely to the lability of phosphohistidines. The hydrolysis rate is likely to vary widely in proteins, but studies have shown a clear difference in the stability of phosphohistidine in acidic solution compared with phosphoserine, phosphothreonine, and phosphotyrosine. In the presence of 1 M HCl at 100 °C, phosphoserine and phosphothreonine are relatively stable and have half-lives of approximately 18 h, and phosphotyrosine has a half-life of 5 h [7]. In contrast, 1- and 3-phosphohistidine have half-lives of 18 and 25 s, respectively [8]. Although histidine and aspartic acid residues are likely the most common sites of phosphorylation in nature, relatively few investigations have looked at autophosphorylation with purified histidine kinases, choosing instead bioinformatic or molecular biological approaches to study the regulation of histidine kinases.

In this article, we present a new tool to study the autophosphorylation of a histidine kinase that uses ATPγS. We found that (i) some histidine kinases will use ATPγS to form stable thiophosphohistidines even when (in some cases) the protein will not form a stable phosphohistidine, (ii) the regulation of histidine kinase activity by other protein domains is similar for both ATP and ATPγS, and (iii) the thiophosphate can be transferred to downstream response regulator proteins.

We demonstrate that histidine kinases tolerate S for O substitution in the catalytic cycle. This produces a product that will be stabilized against nucleophilic attack and hydrolysis relative to the corresponding phosphate. Therefore, thiophosphorylated residues in histidine kinase and response regulator proteins are more stable than their phosphorylated analogues. This fact was recognized by those interested in studying histidine phosphorylation with mass spectrometry and where extraction of phosphorylated proteins from cells is required [9,10]. Thiophosphoaspartic acid is also likely to be more stable to hydrolysis than phosphoaspartic acid given that the majority of the hydrolysis occurs through attack on the

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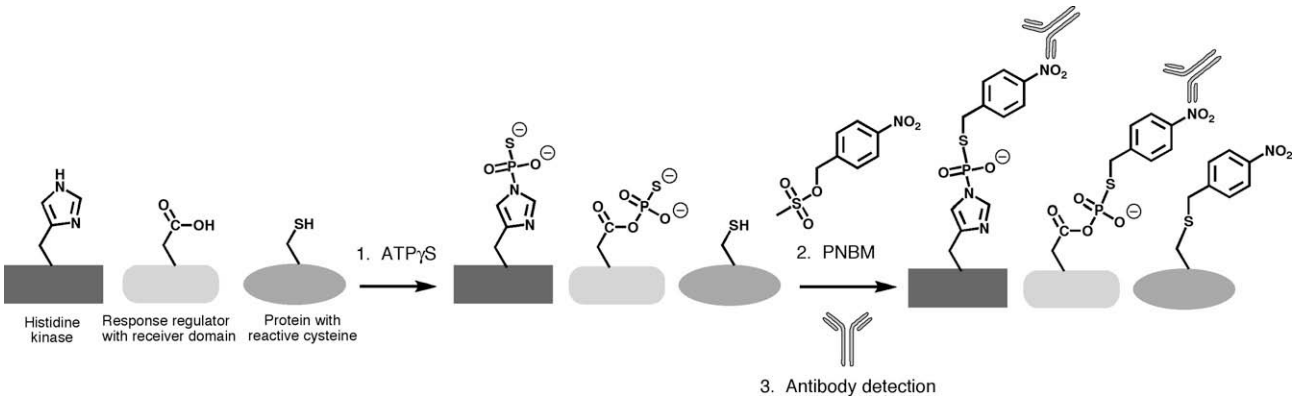
doi:10.1016/j.ab.2009.10.009



Scheme 1. Outline of the method used to detect thiophosphorylated proteins. In step 1, autophosphorylation reactions are initiated with ATPγS and quenched with EDTA. In step 2, PNBM is added to the quenched reactions to alkylate the thiophosphorylated residues. Cross-reactivity occurs with some cysteines. In step 3, an antibody specific for the PNBM-derivatized thiophosphate epitope is used to detect proteins that were thiophosphorylated in step 1.

phosphate rather than the carbonyl carbon of the thiophosphoaspartic acid ester [11].

Previous work by Allen and coworkers has demonstrated the utility of ATPγS as a tool to study serine, threonine, and tyrosine kinase activity [12,13]. After reaction with ATPγS, the kinases and kinase substrates are incubated with *para*-nitrobenzylmesylate (PNBM),[2] which reacts with both cysteine and thiophosphates. An antibody specific for the alkylated thiophosphate is then used to detect the thiophosphate esters. So long as the protein of interest does not have epitopes that cross-react with the antibody, this method will work to selectively detect thiophosphorylated proteins both *in vitro* and *in vivo*. The basic method, as applied to histidine–aspartic acid phosphotransfer pathways, is outlined in Scheme 1.

Materials and methods

Histidine kinase cloning and expression

CheA and NtrB proteins were gifts from Brian Crane (Cornell University) and Sydney Kustu (University of California, Berkeley). *Shewanella oneidensis* H-NOX (heme–nitric oxide oxygen binding proteins)-associated histidine kinase (SO2145) was cloned and expressed as described previously [14]. Other histidine kinase proteins (LPG2458, HCH03701, and VFA0072) were cloned out of genomic DNA and ligated into pET-20b(+) expression vector (Invitrogen) cut with NcoI and XbaI restriction enzymes (NEB) and transformed into *Escherichia coli* DH5α. The cytoplasmic domain of *E. coli* EnvZ (residues 223–450) [15] was cloned out of genomic DNA and ligated into pET-20b(+) vector cut with NdeI and NotI restriction enzymes (NEB). The receiver domain of *E. coli* OmpR (residues 1–128) was cloned out of genomic DNA and inserted into the Gateway entry vector pENTR/SD/D-TOPO using a TOPO cloning kit (Invitrogen). N-Terminal, MBP-tagged OmpR was constructed by transferring the gene into the Gateway destination vector pHMGWA [16] using LR clonase II mix (Invitrogen). Positive transformants of all constructs were screened for on Luria–Bertani (LB) plates containing 100 μg/ml ampicillin, and the DNA sequences were confirmed by sequencing (Elim Biopharmaceuticals). With the exception of CheA and NtrB, all proteins were expressed as follows: *E. coli* BL21(DE3)pLysS cells containing the appropriate plasmid were grown at 37 °C to an OD_{600} of 0.6–0.9, induced with 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), and grown for 16–18 h at 25 °C. Cells were harvested by centrifugation at 7000 rpm (6370*g*) for 15 min in an Avanti J-20 I centrifuge with a JLA 8.1 rotor (Beckman), resuspended in lysis buffer (100 mM sodium phosphate, 250 mM NaCl, 5% glycerol, and 20 mM imidazole, pH 7.9), and lysed with a high-pressure homogenizer (Avestin). Lysate was clarified by centrifugation at 42,000 rpm (200,000*g*) for 1 h in an Optima XL-100K ultracentrifuge with a Ti-45 rotor (Beckman) prior to loading on nickel–nitrilotriacetic acid (Ni–NTA) agarose resin (Qiagen). The Ni–NTA resin was washed with lysis buffer and eluted with elution buffer (250 mM imidazole in 100 mM sodium phosphate, 250 mM NaCl, and 5% glycerol, pH 7.9). Gel filtration chromatography with an S200 26/60 HiLoad Resin column (Pharmacia Biotech) connected to a Biologic HR fast protein liquid chromatography (FPLC) device was used for further purification. The gel filtration column was equilibrated and run in 50 mM triethanolamine (TEA), 50 mM NaCl, and 5% glycerol (pH 7.5) buffer. All proteins were more than 95% pure for assays, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining. Protein concentrations were determined by the method of Bradford [17] and quantitative amino acid analysis.

Histidine kinase autophosphorylation and response regulator assays using ATPγ^{32}P or ATPγ^{35}S

Histidine kinases (1 or 5 μM) and response regulators (5 μM) were mixed with 500 μM ATP (Sigma–Aldrich) plus 10 μCi ATPγ^{32}P (6000 Ci/mmol, Perkin-Elmer) or 500 μM ATPγS (Sigma–Aldrich) plus 10 μCi ATPγ^{35}S (1250 Ci/mMol, Perkin-Elmer) and 10 mM $MgCl_2$ in 50 mM TEA, 50 mM NaCl, and 5% glycerol (pH 7.5) in 25-μl reactions. At endpoints, the reaction was quenched with 5 μl of a 6× concentrated stock of SDS–PAGE running buffer. Proteins in SDS–PAGE running buffer were not boiled because of concerns about the stability of the phosphorylated species. Proteins were separated from nucleotides on 10–20% Tris–glycine SDS–PAGE gels (Invitrogen). Gels were exposed to a phosphorimager plate (Molecular Dynamics) for at least 16 h and imaged using a Typhoon (Molecular Dynamics).

Histidine kinase autothiophosphorylation and response regulator assays using ATPγS

Histidine kinases (1 or 5 μM) and response regulators (5 or 20 μM) were mixed with 500 μM ATPγS and 10 mM $MgCl_2$ in

[2] *Abbreviations used:* PNBM, *para*-nitrobenzylmesylate; H-NOX, heme–nitric oxide/oxygen; LB, Luria–Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni–NTA, nickel–nitrilotriacetic acid; FPLC, fast protein liquid chromatography; TEA, triethanolamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; PBST, phosphate-buffered saline (pH 8.0) containing 0.5% Tween 20; HRP, horseradish peroxidase; NO, nitric oxide.

50 mM TEA, 50 mM NaCl, and 5% glycerol (pH 7.5) in 25-μl reactions. At endpoints, the reaction was quenched with 5 μl of 500 mM ethylenediaminetetraacetic acid (EDTA), and 1.5 μl of PNBM was added from a 50-mM stock in 100% dimethyl sulfoxide (DMSO) to give a final concentration of 1 mM PNBM and 6% DMSO. After allowing the alkylation reaction to proceed for 1.5 h, 6 μl of a 6× concentrated stock of SDS–PAGE gel running buffer was added. Proteins in SDS–PAGE running buffer were not boiled so as to be consistent with the procedure used in autophosphorylation assays. Proteins were separated from other components of the reaction mixture on 10–20% Tris–glycine SDS–PAGE gels (Invitrogen).

Thiophosphohistidine detection assays

Proteins were transferred from SDS–PAGE gels to nitrocellulose membranes (Whatman) and blocked with 5% nonfat dry milk (Carnation) in PBST (phosphate-buffered saline [pH 8.0] containing 0.5% Tween 20) for 1 h at room temperature. Primary antibody specific for the alkylated thiophosphate (Epitomics, monoclonal antibody 51-8) was added at 1:5000 in 5% nonfat dry milk in PBST and incubated with the blot overnight at 4 °C. The blot was then washed three times (for 10 min each) with PBST at 25 °C. Secondary antibody, goat anti-rabbit horseradish peroxidase (HRP, Pierce), was added at 1:1000 also in 5% nonfat dry milk in PBST at 25 °C for 1 h. The blot was again washed three times (for 10 min each) with PBST at 25 °C and was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and imaged using a Fluor-S MultiImager (Bio-Rad). Negative control reactions in which ATPγS was not added or PNBM was not added were run to confirm that there was no cross-reactivity with alkylated cysteines or other protein epitopes.

Results and discussion

The panel of histidine kinases chosen for this study was largely based on previous work to study the regulation of histidine kinases by adjacent H-NOX proteins. In that work, we demonstrated that the H-NOX from *Shewanella oneidensis* regulates the activity of a histidine kinase found in the same operon [14]. Unfortunately, attempts to show similar regulation in proteins from other organisms have proven to be more difficult. Fig. 1 illustrates the fact that many histidine kinases that do not form a stable phosphohistidine will form a stable thiophosphohistidine. For comparison, the well-studied histidine kinases *Thermotoga maritima* CheA and *E. coli* NtrB are shown in lanes 1 and 2, respectively. Lanes 3, 4, 5, and 6 show the H-NOX-associated histidine kinases from *S. oneidensis*, *Legionella pneumophila*, *Hahella chejuensis*, and *Vibrio fischeri*, respectively. Of particular note are the histidine kinases from *V. fischeri* and *H. chejuensis.* Neither of these proteins forms a stable phosphohistidine with ATP, but both form stable thiophosphohistidines with ATPγS. In the case of the *Hahella* kinase, although no stable autophosphorylated protein could be detected, the thiophosphate was stable overnight at 25 °C (see Supplemental Fig. 2 in Supplementary material). Also of interest is the CheA protein from *T. maritima.* It does not accept ATPγS as a substrate (Fig. 1 and Supplemental Fig. 1). Given that the conditions used for autophosphorylation and autothiophosphorylation reactions are identical except for the nucleotide used, the stability of the thiophosphate is likely what allows detection in cases where the phosphorylated protein cannot be detected. In some of the histidine kinases, such as the H-NOX-associated kinase from *S. oneidensis*, the intensity of the signal in the ATPγ^{32}P gel is different from that of the signal in the ATPγS Western blot (Fig. 1). This can be explained either by a difference in the ability of the protein to accept ATPγS rather than ATP or by differences in the degree of PNBM alkylation or antibody recognition of the alkylated thiophosphate in the protein. In the case of the *S. oneidensis* kinase, the differences in Fig. 1 are likely explained by differences in alkylation or recognition of the epitope given that the protein was exceptionally active with ATPγ^{35}S when compared with other proteins in the panel but was much less active in the ATPγS antibody assay (see Supplemental Fig. 1).

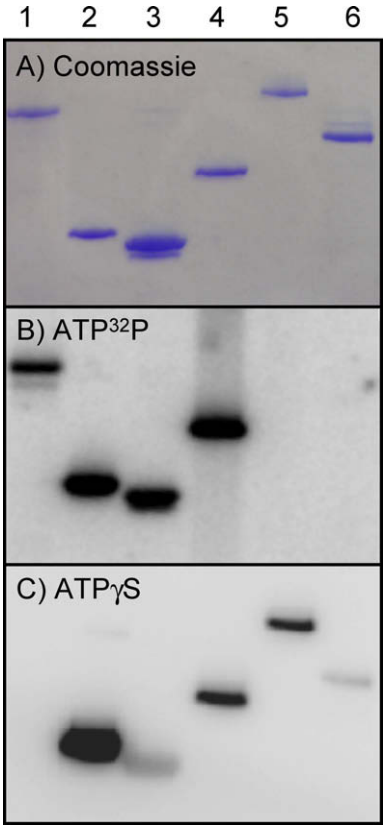


Fig. 1. A panel of six purified histidine kinases were tested using radioactive ATP^{32}P and the ATPγS antibody assay. (A) Coomassie-stained gel of the panel of purified histidine kinases. (B) Phosphorimage of radioactive gel showing the panel of histidine kinases tested using ATP^{32}P. (C) Western blot showing the panel of histidine kinases tested using the ATPγS antibody assay. Lane 1: *T. maritima* CheA; lane 2: *E. coli* NtrB; lanes 3, 4, 5, and 6: H-NOX-associated kinases from *S. oneidensis*, *L. pneumophila*, *H. chejuensis*, and *V. fischeri*, respectively. The molecular weights of the proteins are as follows: *T. maritima* CheA, 75.5 kDa; *E. coli* NtrB, 23.6 kDa; SO2144, 34.9 kDa; LPG2458, 49.2 kDa; HCH03701, 87.4 kDa; VFA0072, 65.3 kDa.

We did not determine which isomers of the thiophosphohistidine were detected in the proteins we tested. However, given that the antibody was initially raised to detect thiophosphate esters of hydroxy amino acids [11,12], the epitope recognized by the antibody is unlikely to include any part of the amino acid. This should permit detection of either 1-thiophosphohistidine or 3-thiophosphohistidine.

It should also be noted that the *V. fischeri* and *H. chejuensis* histidine kinases are hybrid kinases and have a receiver domain that may contribute to the rapid hydrolysis rate. In addition, the aspartic acid residues in the receiver domains of the hybrid kinases may be thiophosphorylated and, therefore, contribute to the overall signal detected for these proteins in the assays. From our experiments, we conclude that because (i) the reactivity of the antibody toward the alkylated thiophosphate may vary in the context of different proteins and (ii) the rate of ATPγS turnover may vary for different proteins, care should be taken when comparing the relative autothiophosphorylation rates between proteins using this assay.

In our previous work with H-NOX/histidine kinase pairs, we found that the H-NOX domain inhibits the autophosphorylation

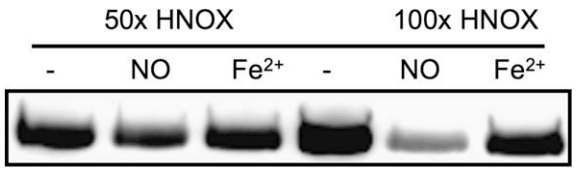


Fig. 2. The autophosphorylation activity of the H-NOX-associated kinase from *H. chejuensis* is inhibited by the addition of H-NOX in the Fe^{2+}–NO ligation state, but to a lesser extent by the Fe^{2+} unligated state. Kinase (1 μM) was incubated for 1.5 h with 500 μM ATPγS and 2.5 mM $MgCl_2$ in the presence or absence of an excess of H-NOX in either the Fe^{2+}–NO ligation state or the Fe^{2+} unligated state. The inhibition of autophosphorylation activity by the NO-bound H-NOX for the *H. chejaensis* kinase is similar to that seen in other H-NOX-associated kinases that are stably phosphorylated on incubation with radioactive ATPγ^{32}P.

of the histidine kinase in the nitric oxide (NO)-ligated state but not in the reduced unligated state (see Ref. [14] and H.K. Carlson, unpublished work). This was further tested for other bacterial H-NOX proteins, and a similar regulation was found for the H-NOX–kinase pair from *H. chejuensis* (Fig. 2). At 100-fold excess of the H-NOX, the reduced unligated state of the protein inhibited the autothiophosphorylation rate of the *H. chejuensis* histidine kinase to a much lesser extent than the NO-ligated state (Fig. 2). Autophosphorylation of the *H. chejaensis* kinase in the presence or absence of the *H. chejaensis* H-NOX protein using ATP was not observed (Fig. 1). This is a clear example of the utility of the ATPγS method in studying the regulatory mechanism of challenging, novel phosphotransfer pathways.

In contrast to many serine, threonine, and tyrosine kinases, histidine kinases autophosphorylate prior to transferring the phosphate to a response regulator. This poses some problems for the ATPγS method to study phosphotransfer. The thiophosphohistidine is expected to be more stable to hydrolysis and might also display sluggish phosphotransfer kinetics. However, the transfer of thiophosphate from the histidine kinase EnvZ to its cognate response regulator OmpR was observed, as was the transfer of thiophosphate from the H-NOX-associated kinase from *H. chejuensis* to the response regulator in the same predicted operon (Fig. 3). Transfer from EnvZ to OmpR was detectable at equal concentrations (5 μM), but higher concentrations of the *H. chejaensis* kinase (20 μM) were needed to observe transfer to its response regulator. A method to selectively detect the downstream partner of a histidine kinase would be broadly useful in the discovery of prokaryotic phosphotransfer pathways. Current methods for determining the cognate response regulator for a histidine kinase require purification of all response regulators from an organism and determination of the kinetic preference for phosphotransfer [18].

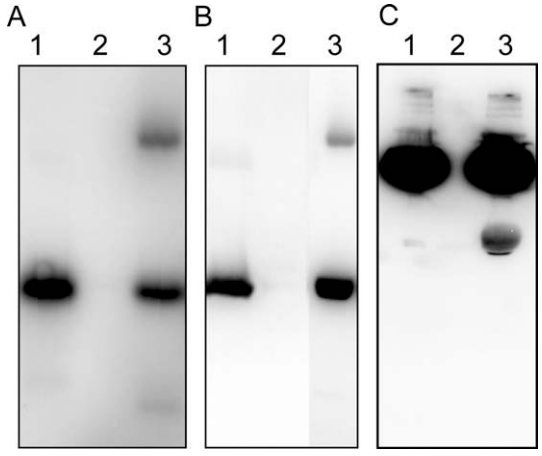


Fig. 3. Thiophosphate transfer from histidine kinases to response regulators. (A) Reaction with ATPγ^{32}P showing phosphate transfer from the histidine kinase EnvZ to MBP-tagged OmpR. Lane 1: EnvZ; lane 2: MBP–OmpR alone; lane 3: MBP–OmpR + EnvZ (5 μM EnvZ and 5 μM MBP–OmpR). (B) Reaction using ATPγS and the antibody assay showing thiophosphate transfer from the histidine kinase EnvZ to MBP-tagged OmpR. Lane 1: EnvZ; lane 2: MBP–OmpR alone; lane 3: MBP–OmpR + EnvZ (5 μM EnvZ and 5 μM MBP–OmpR). (C) Thiophosphate transfer from the H-NOX-associated histidine kinase in *H. chejuensis* to the response regulator found in the same predicted operon. Lane 1: *Hahella* histidine kinase; lane 2: *Hahella* response regulator alone; lane 3: *Hahella* histidine kinase + response regulator (20 μM *Hahella* histidine kinase and 5 μM *Hahella* response regulator).

Conclusion

The assay method described in this article is likely to be broadly useful for studying the activity of histidine kinases—both as an alternative to radioactive ATP and as a means to probe the activity of histidine kinases that do not accept ATP to form stable phosphohistidines. Unlike phosphospecific antibody methods used in eukaryotic kinase studies, where each antibody exhibits specificity for the specific phosphorylated residue based on the neighboring amino acids, the thiophosphate–PNBM-specific antibody is context independent and can be used for detection of the phosphate modification in any protein context. Further work is necessary to determine the utility of this assay for characterizing new phosphotransfer pathways. However, the transfers of thiophosphate from EnvZ to OmpR and between the *Hahella* kinase and the response regulator are very promising results. Further work will also focus on using this technique for the preparation of thiophosphorylated proteins as an alternative to BeF_3 for structural studies or mass spectrometry.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grants GM070671 (to M.A.M.) and EB001987 (to K.M.S.). Thanks go to Epitomics for generously providing antibody 51-8 for these studies. The authors are very grateful to Doug Mitchell, a former member of the Marletta lab who suggested that we try this approach. In addition, we thank members of the Marletta and Shokat labs for stimulating discussion and advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.10.009.

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