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Characterization of specific binding by mass spectrometry: Associations of *E. coli* citrate synthase with NADH and 2-azidoATP

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ABSTRACT

Type II citrate synthases (CSs), found in gram-negative bacteria such as *Escherichia coli*, are subject to strong and specific allosteric inhibition by NADH. Crystallographic studies have shown that the NADH binding sites are located at macromolecular contacts, so that tight binding requires the intact hexameric structure. Although other adenylates, such as 5′-AMP and ATP, are known to influence CS function by inhibiting NADH binding, the exact mechanism of inhibition is currently unknown. In the present study, we have used mass spectrometry to study the adenylate-binding properties of CS by ESI and MALDI-based techniques. More specifically, to investigate where adenylates bind, we have used the photoaffinity label, 2-azidoATP, to label *E. coli* CS under non-denaturing conditions for MS analysis of tryptic peptides. Different MS techniques are used to show that tryptic peptide T17 (AA168–177) of the CS sequence known to contribute to NADH binding, disappears after photolabeling, while a new photoaffinity-labeled peptide, with a mass 520 Da than that of T17, appears instead. Another new peptide, corresponding to photolabeled peptide T16–18 (a combined peptide arising from incomplete digestion of the same region) has also been detected. By CE-ESI-TOF-MS, ESI-Q^3, MALDI-TOF and analysis of complex stoichiometry we conclude that ATP/ATP-analogs, and presumably the whole series of adenylates potentiate CS and the tricarboxylic acid cycle through the identified site of NADH regulation.

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1. Introduction

Citrate synthase (CS), an enzyme found in nearly all organisms, catalyzes the condensation of acetyl-coenzyme A and oxaloacetate to form citrate, the entry point of carbon into the tricarboxylic acid cycle. In most organisms, the enzyme is a dimer of identical subunits, with no regulatory properties; this has been designated Type I CS. In gram-negative bacteria such as *Escherichia coli*, CS is a hexamer (a trimer of dimers), designated Type II CS, which is strongly and specifically inhibited by the biological reducing agent, NADH, by an allosteric mechanism [1].

For one Type II CS, that from *E. coli*, detailed structural information is available from crystallographic studies of the solid state [2,3]. The NADH binding sites are located at the dimer–dimer interfaces, in six approximately equivalent positions, some 20 Å from the nearest active sites. The binding site pockets are overall cationic, and involve interactions between NADH and several amino acid side chains in the 106–206 stretch. In *E. coli*, CS monomers comprise 426 amino acids (47,887 Da), and the enzyme is an equilibrium mixture of dimers (95,774 Da) and hexamers (287,322 Da). The dimer–hexamer equilibrium shifts towards hexamers in the presence of NADH, as a result of NADH binding specifically to the hexameric form [4]. In-solution experiments involving fluorescence [5] and ESI-MS [4] have been conducted to determine K_D values for the CS–NADH system. Fluorescence experiments indicated a K_D of 1.6 ± 0.1 μM for binding of NADH to CS [5]. Detailed analysis of mass spectra obtained from the titration of CS with NADH [3] revealed three types of binding: (i) tight, specific binding of 6 NADH molecules per CS hexamer, $K_D = 1.1 \pm 0.2$ μM; (ii) weak, non-specific binding of 12 NADH per CS hexamer, $K_D = 155 \pm 19$ μM; (iii) weak, non-specific binding of 2 NADH per CS dimer, $K_D = 28.3 \pm 3.4$ μM [4].

NADH is a derivative of adenylic acid, and several other adenylates, such as AMP, ADP, ATP, and ADP-ribose, have been shown to inhibit NADH binding. It has been presumed that these molecules also bind at the NADH site, though they are not allosteric inhibitors [5,6]. It is of interest to establish whether the other adenylates do indeed bind at the NADH site. One way to approach this question is photoaffinity labeling. This type of strategy utilizes a photoactive

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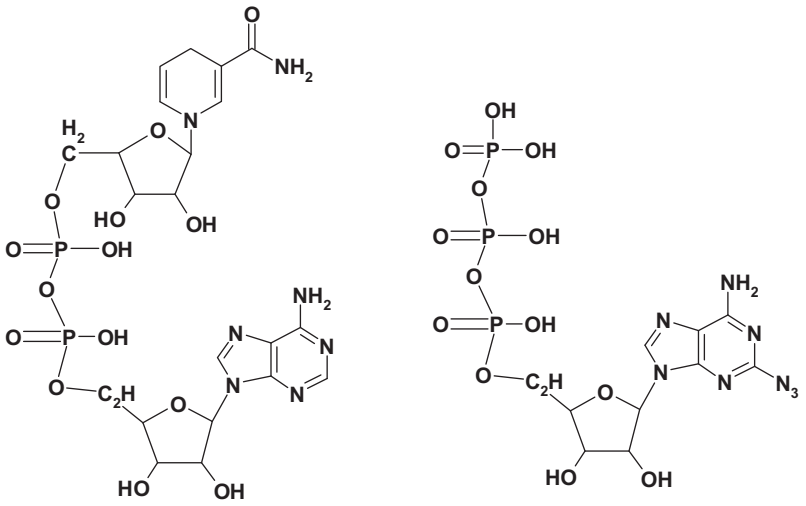

Fig. 1. Structures of NADH and 2-azidoATP.

ligand analog that produces a reactive species which irreversibly binds to a receptor when irradiated with UV light. Photoaffinity labeling allows for specific non-covalent associations to be established in the natural aqueous phase prior to covalent modification, increasing the probability of identifying the region of interest.

Numerous studies and reviews describing photoaffinity labeling have been published, showing the utility of the method in identifying ligand binding sites of various enzymes [e.g. [7–9]]. This method requires careful selection of the probe and incubation mixture in order to maximize specific non-covalent associations with the receptor and to minimize non-specific labeling of the enzyme and/or any other material. Most labels used are azide analogs of substrates or inhibitors capable of binding to the enzyme; upon UV irradiation, the azide group loses molecular nitrogen, and a highly reactive nitrene is released which attaches covalently to a nearby part of the protein. Photolabeling experiments typically use ^{32}P labels. Radioactive peptides are separated by HPLC, followed by sequencing to identify the location of the photolabel.

In the work described in this report, photoaffinity labeling was employed to better characterize the amino acid sequence associated with the allosteric region of *E. coli* CS. An azide analog of NADH was considered, however no probe based on NADH was commercially available. The photoaffinity label 2-azidoNAD$^+$ was commercially available, however studies have shown that NAD$^+$ shows no inhibitory effects and is at best only a weak competitive inhibitor to NADH binding [5,6]. Instead, we evaluated 2-azidoATP and 8-azidoATP, two commercially available azido analogs of one of the adenylates thought to bind at the NADH sites. The literature K_D value for ATP binding to CS is $46 \pm 5\,\mu$M [5], suggesting the possibility that these photolabels might be specific for the NADH site. Fig. 1 shows the structures of 2-azidoATP and NADH.

In this work, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) were employed to detect photolabeled and unmodified CS. Quantitative measurements on the formation of non-covalent unlabeled CS–NADH complexes were also performed and compared with previous results [4]. ESI- and MALDI-MS spectra were acquired for the tryptic peptides of labeled and unlabeled CS, in order to elucidate the location of the attachment sites. These MS techniques allowed for the use of non-radiolabeled photoaffinity probes, avoiding the safety protocols that are required in the presence of radioactivity. Tryptic peptides were also characterized by on-line capillary electrophoresis (CE) coupled to ESI-MS detection.

2. Experimental

2.1. Chemicals

Ammonium bicarbonate, sinapinic acid and TCPK-treated trypsin were obtained from Sigma Chemicals (St. Louis, MO). Reagents Tris, EDTA, KCl, NADH (di-sodium salt) were also purchased from Sigma. The photolabels, 2- and 8-azidoATP, were obtained from ALT Inc., Lexington, KY. Radioactive (^{32}P) versions of these reagents were obtained from ICN Pharmaceuticals, Costa Mesa, CA. All solutions were prepared in deionized, filtered water from a Barnstead Nanopure water filtration system supplied by a reversed osmosis feedstock.

2.2. Preparation of E. coli citrate synthase

The enzyme under investigation was obtained and purified as described by Duckworth and Bell [10]. CS samples, initially in 20 mM Tris, 1 mM EDTA and 50 mM KCl, were dialyzed against 20 mM ammonium bicarbonate using a membrane with 12,000–14,000 molecular weight cutoff (Spectra/Por, Laguna Hills, CA).

2.3. Study of citrate synthase/NADH non-covalent adducts

Four CS samples, each 4.45×10^{-4} M in 20 mM ammonium bicarbonate, were incubated with NADH, at concentrations of the latter corresponding to 1×, 5×, 10× and 20× that of CS. The incubation time was 20 min, at room temperature. In this paper, ESI-MS of these samples was performed on a Quattro-LC instrument (Micromass, UK) as described below.

2.4. Assessment of photolabeling conditions

Preliminary characterization of 2- or 8-azidoATP binding to CS was assessed using γ ^{32}P radioactive photolabels. The labels were prepared in a volume of CS 0.4 mg/L solution in 20 mM ammonium bicarbonate to give final photolabel concentrations ranging from 0 to 400 mM.

To assess the conservation of the whole radioactive photolabel, some experiments used a mixture of radioactive and non-radioactive label at a known molar ratio. These mixtures were incubated on ice for 2 min, then exposed to UV light from a Mineralight R-52G lamp with the filter removed, at a distance

of approximately 5 cm, for the desired length of time. The samples were then immediately mixed with SDS PAGE sample loading buffer (0.3 M TrisCl, 3.6 mM 2-mercaptoethanol, 10% SDS, 50% glycerol, with bromophenol blue), vortexed, and loaded onto 8% SDS PAGE gels, without boiling the samples, as this can cause loss of radiolabel [11]. After electrophoresis, staining and destaining, gels were wrapped in plastic and exposed to X-ray film overnight. Quantitation of photolabel incorporation was done by liquid scintillation counting of the CS gel bands. Two standards containing 75–100 μg of CS were run on the outside lanes of each gel, and showed prominently after staining. Using these visible standards as guides, sample bands were excised as gel slices. As controls, one standard band, and a comparable piece from an unused lane were also collected. Gel slices were immersed in 5 mL of Amersharn Aqueous Counting Scintillant, and counted immediately in an LKB-Wallac 1215 Rackbeta II Liquid Scintillation Counter. Readings in counts per minute were converted to % photoincorporation as described by Bhattacharyya et al. [11]. Once the optimized conditions for photoaffinity labeling of CS were established, those conditions were used for the modification of the enzyme with non-radioactive probe for tryptic mapping by MS, as described below.

2.5. Specifics of non-radioactive photolabeling reaction

Citrate synthase (11 nmol in 12.5 μL) was incubated with 8.9×10^{-3} M 2-azidoATP (10-fold the amount of CS) in low salt buffer (2 mM Tris, 0.2 mM EDTA, 10 mM KCl) at pH 7.8 for 30 min at room temperature. The sample was then transferred and sealed in a quartz micro-cell (Markson Science, Del Mar, CA) and submerged in a water bath maintained at 14 °C. The sample was further subjected to UV radiation from a tungsten–mercury light source (Hanovia, Union, NJ) for 20 min. Salts and unbound photolabel were removed using a Micron™ molecular weight 10,000 cutoff centrifuge filter (Millipore, Bedford, MA) and rinsed with 20 mM ammonium bicarbonate.

2.6. Tryptic digestion of labeled and unlabeled CS samples

Citrate synthase and photolabeled CS (1.0 mg/mL in 20 mM Tris–HCl and 1 mM EDTA, pH 7.8) were separately incubated with 0.01 mg/mL of trypsin. Two different procedures were used for different batches of samples: (i) digestion took place for 3 h at 37 °C (Batch 1), and (ii) digestion took place for 18 h at room temperature (Batch 2). The tryptic peptides were then concentrated with C18 Ziptip™ (Millipore) micropipette tips prior to MS analysis.

2.7. Electrospray ionization mass spectrometry

Spectra during direct infusion and capillary electrophoresis (CE) experiments were obtained on ESI-TOF III, a mass spectrometer built in-house in the Physics Department, University of Manitoba, as described elsewhere [12–14]. Data were acquired and processed using TOFMA software, written in-house. CE/MS separations were conducted as described previously [13]. Other ESI-MS spectra were obtained on a Quattro-LC mass spectrometer (Micromass, UK) equipped with a Z-Spray™ ionization source. Spectral acquisition and analysis were conducted using Masslynx 3.0 software. All ESI data were obtained in the positive mode using 3.6 kV spray potential and 40 V declustering (cone) voltage. The source block was maintained at 130 °C and the desolvation gas, nitrogen, was heated at 110 °C. A 50:50 mixture of acetonitrile–water with 0.1% acetic acid was used as carrier solvent, at a flow rate of 10 μL/min.

2.8. Matrix-assisted laser desorption/ionization mass spectrometry

Spectra were obtained on a Biflex-IV instrument (Bruker Daltonics, Billerica, MA) with sinapinic acid as the matrix. The instrument was operated in linear mode for molecular weight measurements of intact CS and photolabeled-CS, and in the reflecting mode for measurements on tryptic digests. Spectra were acquired using Flexcontrol™ software and processed with XTOF™ software.

3. Results and discussion

3.1. E. coli citrate synthase by mass spectrometry

Previous MS studies have shown that *E. coli* CS in solution is mainly a mixture of dimers and hexamers [4]. The evidence consisted of ESI-MS spectra obtained on a custom-built instrument, TOF-III, available in the Physics Department at the University of Manitoba [4,12]. The extended mass range of this instrument along with its soft ionization characteristics allowed the intact non-covalent complexes of CS to be detected and quantified [4]. Triple quadrupole instruments, like the one used here, are unable to perform detection of such large entities due to their limited m/z range (m/z 0–2000). CS complexes were observed as multiply protonated (e.g., >$[M+40H]^{40+}$) resulting from the electrostatic dissociation of CS hexameric/dimeric complexes, and the detection of CS was mainly in the monomeric form. Fig. 2 shows a) ESI-MS and b) linear MALDI-TOF measurements of the molecular weight of CS monomers, which produced signals at masses within 20 ppm of the predicted values.

3.2. Interactions between CS and NADH

Previously it was determined that NADH preferentially interacts with the allosteric site of CS, particularly in the hexameric form [4]. Previous attempts to study enzyme–substrate binding of the same system using an ESI-TOF instrument [15] were inconclusive as there was addition of multiple NADH molecules to CS with no specific number being predominant. Increasing the cone (declustering) voltage would break down the dimers into monomers which still exhibited aberrant NADH attachment patterns. In the present work, interactions between CS and NADH were studied with the Quattro-LC mass spectrometer in order to establish the feasibility of detection of complexes prior to photolabeling experiments. As already stated, Quattro-LC conditions did not make it possible to detect adducts of NADH on CS dimers and hexamers. Z-Spray™ ionization produced a mixture of what appeared to be free monomers and monomers bound to a single NADH moiety (665 u). Adducts with more than one NADH molecule were not observed, which suggests specificity in the CS + NADH observed. To test whether adduct formation was concentration dependent, solutions with NADH concentrations corresponding to 1, 5, 10 and 20 times the CS concentration (4.45×10^{-4} M) in 20 mM ammonium bicarbonate were prepared and examined by ESI-MS on the Quattro-LC. Fig. 3 shows the corresponding raw and recalculated spectra. Integration of peak areas corresponding to CS and CS + NADH indicated that 50% of subunits were available for binding. A K_D value of the order of a few mM was approximately suggested from these data, which represents a much weaker interaction than the allosteric binding of NADH to CS hexamers (K_D = 1.2 μM), but it is in the range of the K_D value measured by Ayed et al. for the interaction of NADH with CS dimers [4]. This suggests that either the dissociation of "1 NADH per CS dimer" complexes is the origin of free and associated monomers observed here with the Quattro-LC electrospray instrument or that there is a large extent of non-specific binding

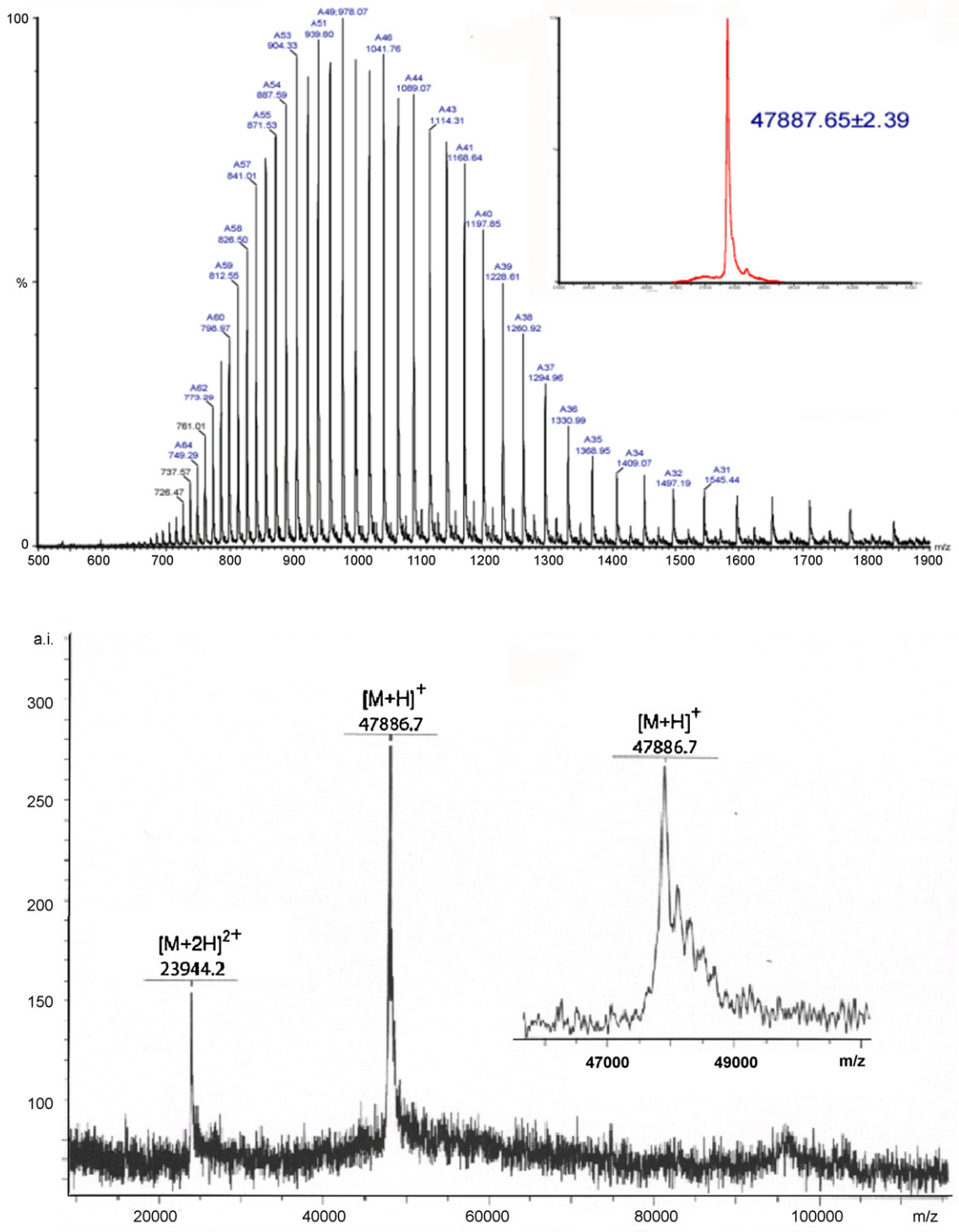

Fig. 2. Mass spectra of citrate synthase a) ESI-MS on the Quattro-LC instrument, b) MALDI-TOF on the Biflex-IV spectrometer. Calculated average molecular weight of citrate synthase: 47,887 Da.

occurring. There is however caution to be paid when indirectly assessing the fragmentation behavior of larger complexes using a low *m*/*z* range instrument due to possible artifacts, as reported by Abzalimov et al. in the case of hemoglobin [16].

In order to further investigate the specificity of CS + NADH binding within the "CS_2 + NADH" form, two series of experiments were performed (results not shown): in the first instance CS was denatured with 15% acetic acid, to ensure the presence of only monomers. Then a solution with 30-fold concentration excess of NADH was added, and the mixture was analyzed by ESI-MS. The spectrum showed a predominance of unbound CS with no measurable adducts formed. This suggested that CS monomers are not likely to form NADH adducts. Then, CS and 30-fold NADH were mixed, prior to adding 15% acetic acid as a denaturant. In this case adducts of one NADH molecule were observed, as well as unattached CS monomers, suggesting that even in acidic denaturing conditions, some of the "CS_2 + NADH" binding is preserved prior to MS measurements.

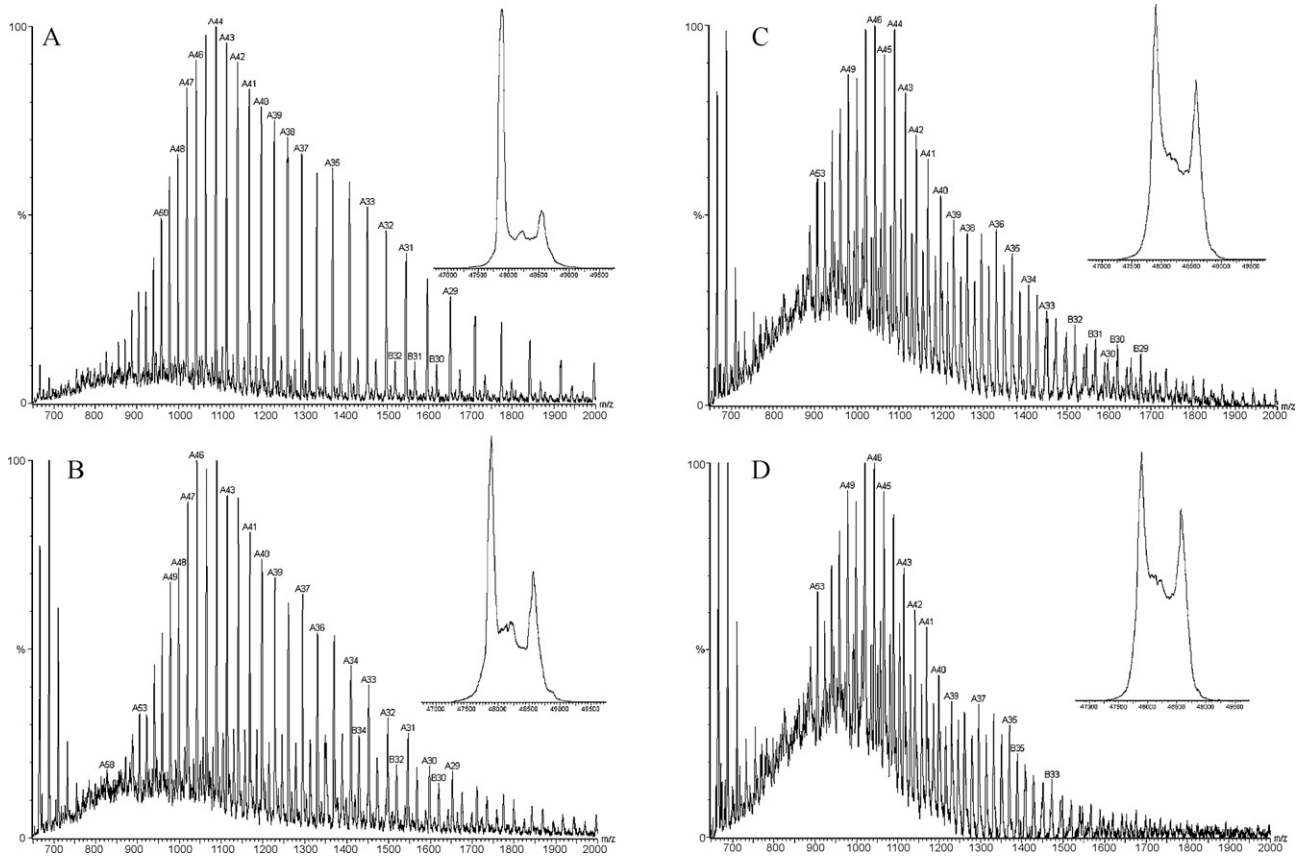

Fig. 3. ESI spectra of CS, incubated with increasing concentrations of NADH, in 20 mM ammonium bicarbonate.

3.3. Establishing optimal conditions for photolabeling

As potential photoaffinity labeling reagents for the NADH binding sites of *E. coli* CS, both azido derivatives of ATP, 2-azido and 8-azidoATP were chosen, as they are commercially available and relatively stable [17]. In previous work, it has been shown that ATP itself and a number of other derivatives of adenylic acid compete with NADH for the binding site, although only NADH itself is an allosteric inhibitor [5,6]. The degree of incorporation of each photoaffinity label was measured by incorporation of γ-^{32}P-labeled azido nucleotide; the position of the label, at the extremity farthest from the reactive azido group, means that incorporation of only complete nucleotide units will be measured.

After optimizing the photoaffinity labeling conditions with 100 μM photoaffinity reagents, the dependence of labeling on reagent concentration was determined. Both azido nucleotides showed saturation, indicating that the labeling requires formation of a CS-reagent complex, and apparent K_D values of about 100 μM and 75 μM were measured for 2-azidoATP and 8-azidoATP, respectively (data not shown, available from [15]). These values may be compared with the previously reported K_D value of 46 ± 5 μM for ATP itself [5,6]. Much more radioactivity was incorporated using the 2-azido reagent (0.35 ± 0.05 moles of label per CS subunit) than the 8-azido isomer (0.07 ± 0.02 moles/subunit), when near-saturating amounts of label (400 μM) were used. Thus, 2-azidoATP is a better labeling reagent in this system. These values could not be increased further by using an even higher concentration of reagent, or by prolonging the reaction time. It was possible, to increase the total incorporation of label, however, by adding a fresh portion of reagent and irradiating again. The highest degree of incorporation, obtained with three rounds of labeling, resulted in attachment of 0.63 moles/subunit to CS protein in the case of 2-azidoATP, but only 0.14 moles/subunit in the case of 8-azidoATP.

To investigate whether the label is specific to the allosteric NADH site, NADH was tested for its ability to inhibit the incorporation of label. High levels of NADH (200 μM, more than 100 × the K_D value of 1.6 μM for NADH binding) reduced labeling from 2-azidoATP by 70%, and labeling from 8-azidoATP by 42% (data not shown, available from [15]). Thus, a large portion of labeling by both reagents is apparently specific for the NADH site.

In all these experiments, 2-azidoATP performed better than the 8-azido isomer: the total incorporation of label was much better, and the specificity for the NADH site was apparently also better. Since the two photolabels have similar affinities for CS protein, the better labeling by the 2-azido isomer may mean that a nitrene group at the 2-position, rather than the 8-position, is more favorably located for reaction with a susceptible amino acid side chain. Therefore, all subsequent experiments were done using protein that had been photolabeled using 2-azidoATP. All samples were subjected to three rounds of labeling, but the non-radioactive reagent was used. Following incubation, the removal of excess unbound label by dialysis was important to prevent further possible non-covalent associations and non-specific chemical reactions. Photochemical quenchers often used in photoaffinity studies, such as dithiothreitol (DTT) and sodium dodecyl sulfate (SDS), were avoided to reduce any interference with MS analysis.

Mass spectrometric investigation of the labeled CS was conducted in several ways. First, the Manitoba III ESI-TOF instrument was used for direct analysis of labeled protein, and also for analysis of tryptic digestion products by sample infusion. Assuming that the label formed a direct nitrene insertion bond within an amino acid, the mass shift produced by the addition of fully protonated 2-azidoATP would be 520 Da. ESI-TOF experiments performed on

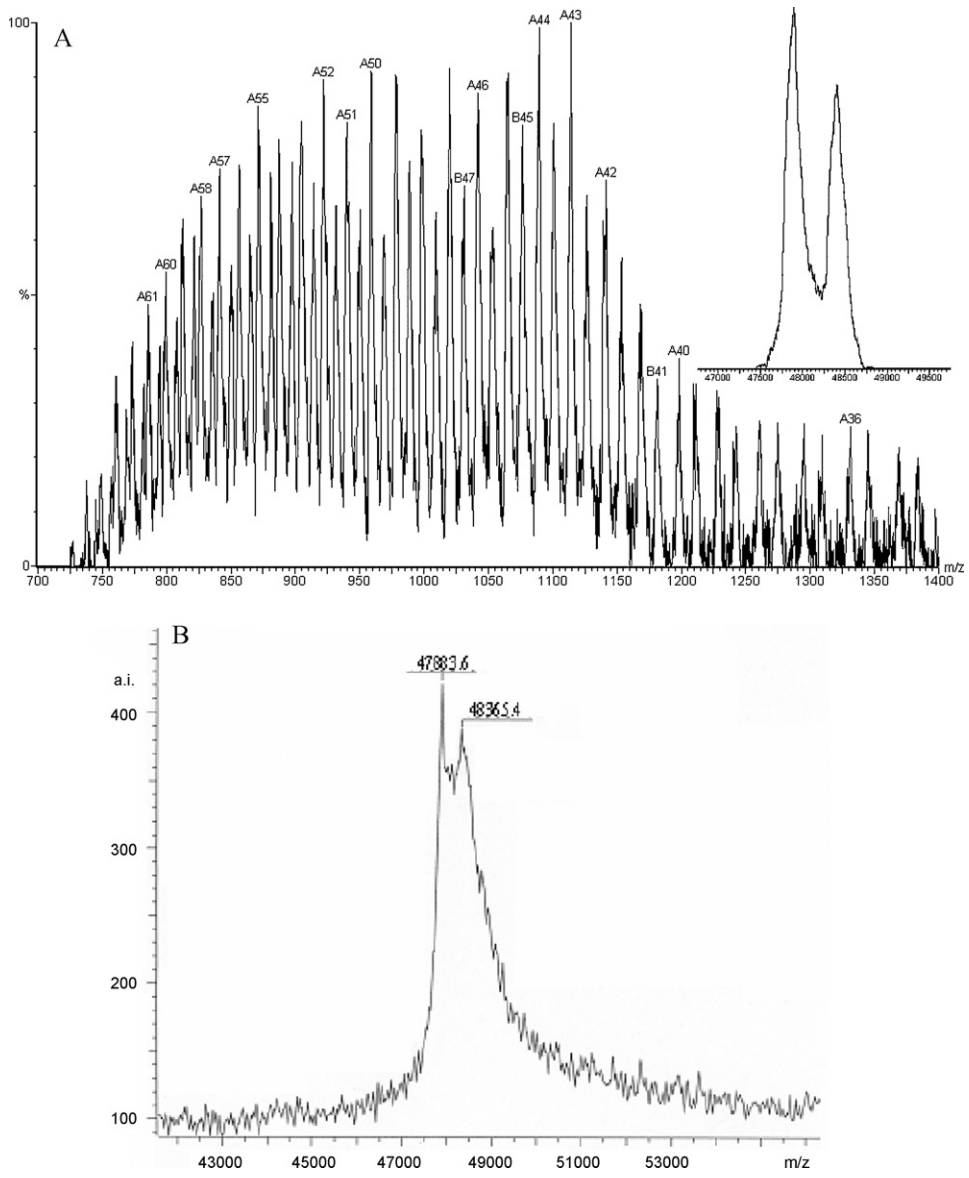

Fig. 4. Mass spectra of citrate synthase, and citrate synthase modified with 2-azidoATP. Calculated molecular weight: 48,407 Da. a) Electrospray (inset: recalculated data) and b) MALDI-TOF.

the Manitoba III instrument allowed us to detect association of up to eight units of 520 Da mass with the CS dimer, under non-denaturing conditions (data not shown, available from [15]). While this result suggested that binding of the photoaffinity nucleotide is non-specific, it did not distinguish between covalent binding (photolabeling) and non-covalent association with excess reagent still present in the mixture. Unlike NADH itself, 2-azidoATP did not seem to shift the multimer equilibrium towards the production of hexamers.

For ESI-MS experiments on the Quattro-LC spectrometer, excess reagent was carefully removed and the proteins were denatured using 15% acetic acid. Only two mass species were found, corresponding to unlabeled CS monomer (measured mass, 47,888 ± 2 Da) and CS monomer with a single photolabel attached (measured mass, 48,400 ± 13 Da; calculated, 38,408 Da) (Fig. 4a). Fig. 4b shows a linear mode MALDI-TOF spectrum of the same sample, also showing the addition of zero or one photolabel molecule per CS. Both experiments show that slightly less than half the protein has been labeled, consistent with what was expected from the optimization work using ^{32}P-labeled reagent.

The mixture of modified/non-modified protein was subjected to proteolytic digestion with trypsin, thus generating peptides suitable for mass spectral measurements. Analyses of the tryptic peptides from both unmodified and photolabeled sample were compared, in search of new peaks of modified peptides corresponding to the mass of a theoretical proteolytic peptide plus the photolabel (+520 Da.). These experiments were conducted on two different batches of CS that had been prepared, photolabeled and digested at different times. Peptides from the first batch were analyzed using direct infusion ESI-TOF-MS and on-line CE/ESI-TOF-MS, while those in the second batch were examined using MALDI-TOF-MS.

On-line capillary electrophoresis–mass spectrometry (CE/MS) of peptides from labeled vs. unlabeled CS showed one major difference between electropherograms: tryptic peptide T17 (residues 168–177) was absent from the photolabeled sample. See Fig. 5 for location of the peptides in the protein and Fig. 6a for the selected ion electropherograms and spectrum. Although this was not a positive identification result, the absence of this peak suggested that T17 might have acquired the intact triphosphate photolabel, and thus

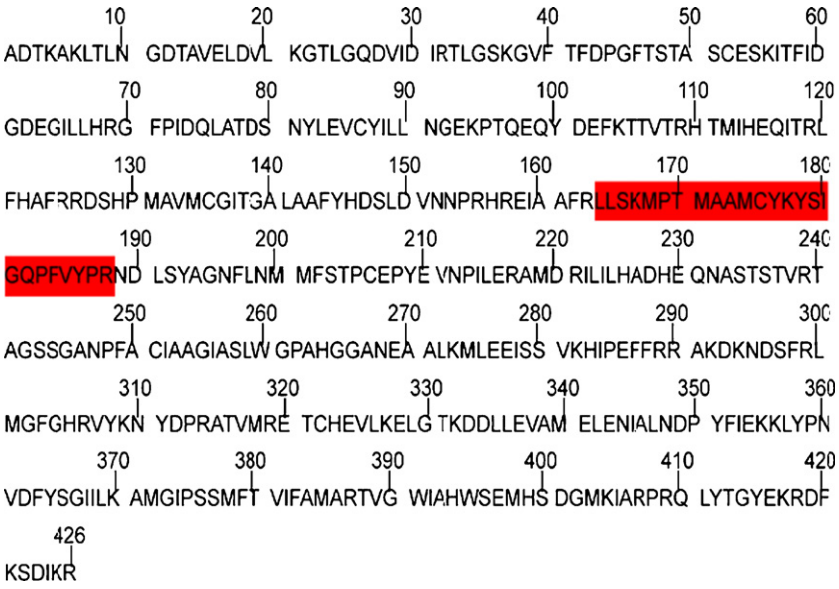

Fig. 5. Amino acid sequence of citrate synthase, with peptides detected as covalently modified (T16–18) highlighted.

might bear a high-density negative charge and not be observed in positive mode. If present in the tryptic digestion mixture, 2-azidoATP-T17 would thus have migrated differently in the capillary. An inspection of the electropherograms to find an earlier peak corresponding to the labeled peptide was inconclusive. The peptide map of CS gave complete coverage for the all 45 expected peptides (T1–T45) of the unlabeled sample, while for the labeled sample, only T17 was missing (Fig. 6b). Direct-infusion analysis of the tryptic digests by positive mode ESI-MS studies reinforced the previous conclusions. In this experiment, the photolabeled sample showed the presence of a new peak at m/z 1666.8, 520 Da heavier than T17 (1146.5 Da), and therefore possibly corresponding to photolabeled T17 or at m/z 1666.8. This novel peak was absent from the control sample (Fig. 7).

Comparison of MALDI spectra in the positive ionization mode only helped to emphasize the absence of unlabeled T17 in the modified Batch 1 of CS sample (Fig. 8). In the negative mode however, the tryptic peptides from Batch 2 of CS produced another difference. Fig. 9 shows comparisons of a) full negative-mode MALDI-TOF-MS spectra of tryptic peptides of photolabeled CS (top trace), unlabeled CS (bottom trace) and b) partial views of the same spectra.

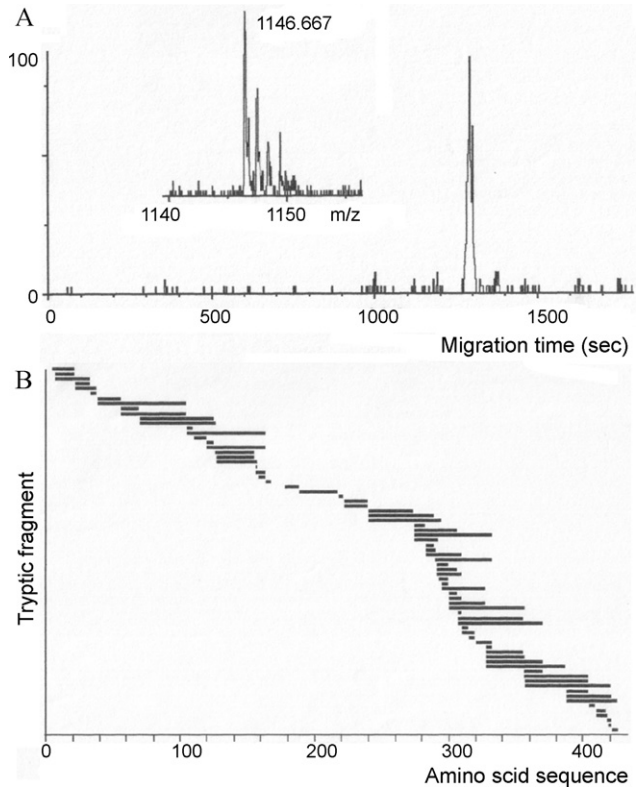

Fig. 6. CE/ESI-TOF-MS of tryptic peptides of unlabeled citrate synthase. a) Selected ion electropherogram of the $[M+H]^+$ ions of T17, m/z 1146.7. This peak was absent from the electrophorogram obtained for the labeled CS sample, which constituted the only observable difference between both sets of results. b) Tryptic peptide map of photolabeled citrate synthase obtained by CE/ESI-TOF-MS. Complete sequence coverage with the exception of T17.

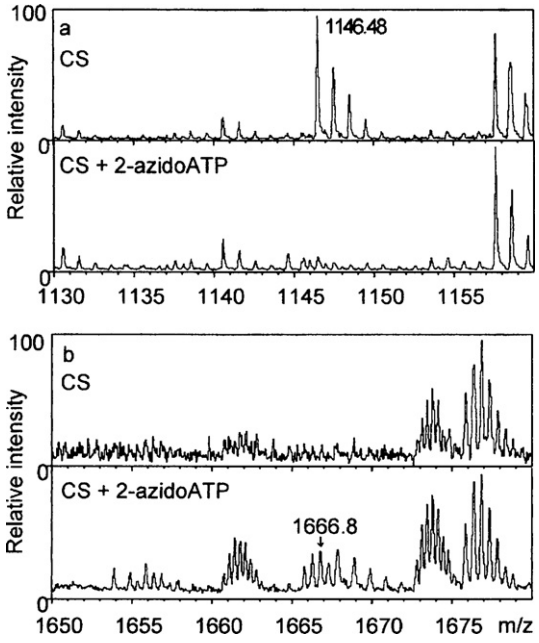

Fig. 7. ESI-TOF mass spectra of tryptic peptides of citrate synthase and 2-azidoATP modified citrate synthase. a) Region of $[M+H]^+$ of T17 and b) region where photolabeled T17 is observed.

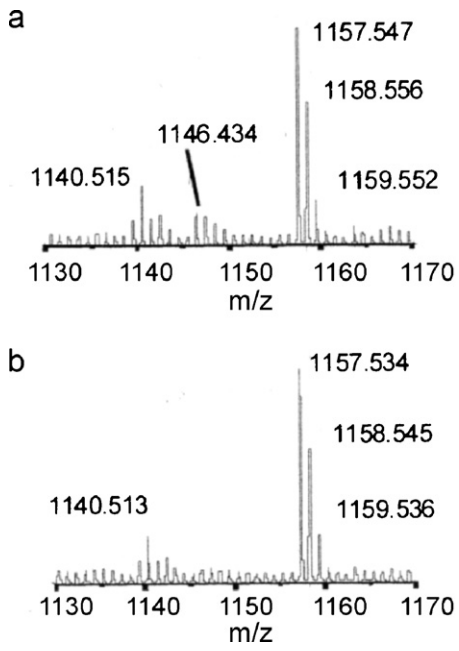

Fig. 8. Positive mode MALDI-TOF mass spectra of tryptic peptides of a) citrate synthase and b) 2-azidoATP modified citrate synthase. Region of $[M+H]^+$ of T17.

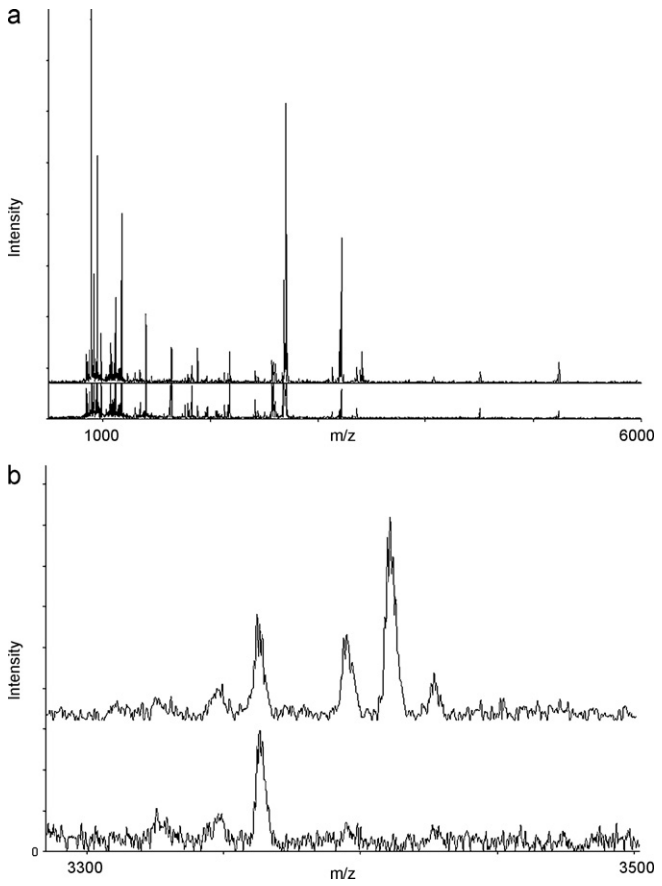

Fig. 9. Negative mode MALDI-TOF mass spectra of tryptic digests of citrate synthase (bottom traces) and 2-azidoATP modified citrate synthase (top traces). a) Full spectra and b) region of photolabeled T16–18.

At *m*/*z* 3413.19, a peak corresponding to amino acids 164–188 (T16–18) plus the photolabel is observed in the top trace of both a) and b).

Photolabeled peptides T17 and T16–18 are still identified only by mass, and have not been sequenced to confirm their identity, and to locate the point or points of attachment of the label. Tandem MS experiments would be necessary to do this. Such experiments have been attempted, but low signal prevented conclusive interpretation of the spectra. There are several possible reasons for this. First, the nitrene generated by the photolabeling procedure is extremely reactive, and it may not always attach to the same amino acid side chain—that is, the targeting method (the ATP moiety of 2-azidoATP) is specific but the modification method (the azido moiety) is not. In fact, multiple sites of incorporation could help to better define a particular binding site. However, MS/MS spectra on an isobaric peptide labeled on different amino acids would be difficult to interpret because of complex superimposed fragmentation patterns. Moreover, the labile nature of phosphate groups during ionization and collision induces dissociation. This multiply phosphorylated modification is anticipated to be exceptionally labile, more so than incorporation of a single phosphate moiety [18]. In a few studies, MS/MS has enabled the identification of photolabeled amino acids, but these reactions rarely lead to only one product and the simpler the label, the clearer results are. For instance, Arevalo et al. [19] have used azialcohols as photolabels to investigate an alcohol binding site in the human protein L1. These photolabels are small molecules with no negative groups, which tended to enhance the ionization efficiency of peptides and even in that situation there were still questions about how many residues reacted and about their positive identification.

T17 and T16–18 are composed of amino acids 164–188 several of which, according to the X-ray crystal structures of CS [2] and of CS interacting with NADH [3], contribute to the NADH binding site. Our evidence that 2-azidoATP can photolabel these peptides, then, is consistent with the belief that this photolabel, and presumably the entire series of structurally related adenylates, bind to the NADH site, as previously surmised.

4. Conclusion

Our mass spectrometric results suggest that the attempt to replace the natural allosteric inhibitor, NADH, with the photolabel 2-azidoATP was successful and we demonstrate for the first time the inhibition of NADH binding to CS by ATP and adenylate analogs occurs through a competitive mechanism. The peptides modified by the photolabel, T16–18, contain amino acid residues which contribute to the allosteric site (e.g., G181 and Q182) as characterized earlier by crystallography. Due to the non-specific binding properties of azido compounds, it would have been expected to find a photolabel at different locations along the amino acids of T16–18. However the combination of crystallographic and mass spectrometric data suggests that there are only a few possible labeling sites. Tandem MS experiments, if they become possible, would be required to establish the position(s) of the label unequivocally. As a binding pocket formed by multiple amino acids would be necessary for NADH adenylate binding to occur, however, it is not expected that a uniform population of molecular ions would be formed.

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