Synthesis of Caged NAD(P)$^+$ Coenzymes: Photorelease of NADP$^+$

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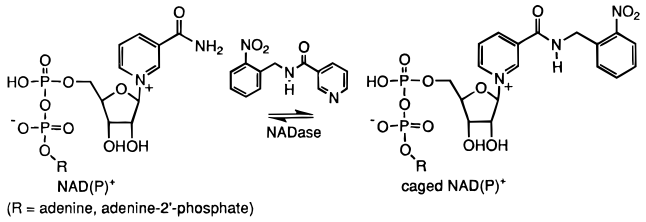
The initiation of enzyme activity can be accomplished through the photolytic release of essential substrates or cofactors from biologically inactive "caged" precursors. There is a growing list of cofactors and substrates which have been caged.[1] These include ATP,[2] GTP,[3] cAMP,[4] Ca $^{2+}$,[5] and several neurotransmitters.[6]

The emerging field of time-resolved crystallography has utilized caged molecules to rapidly initiate catalysis within crystals.[7] Complexation of caged GTP with H-Ras P21 was one of the first examples of such an approach.[3] Exposure of a crystallized caged GTP H-Ras P21 complex to ultraviolet light followed by rapid X-ray crystallographic data collection using synchrotron radiation provided a real-time picture of the catalytic cycle. More recently, crystalline isocitrate dehydrogenase was activated via the photorelease of caged isocitrate.[8]

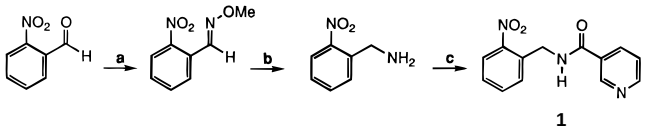
Our current investigations have involved the development of caged NAD(P)$^+$ analogs for use in time-resolved crystallographic studies. The nicotinamide dependent oxido-reductases comprise some one-sixth of all known enzymes, but general methods for their photoactivation are lacking. To date there have been no examples of caged NAD$^+$ or NADP$^+$ analogs. We now report the synthesis of both NAD$^+$ and NADP$^+$ modified at the carboxamide nitrogen with a photolabile *o*-nitrobenzyl group. The use of *o*-nitrobenzyl groups for the photorelease of the carboxamide functionality has been previously demonstrated under aqueous conditions for several biologically active amides, including glutamine and asparagine.[6a] The rate of release of *o*-nitrobenzyl groups under aqueous conditions is typically pH dependent.[1,6a] The quantum efficency of product release is influenced by substitents at either the nitrobenzyl benzylic or aromatic groups.[1]

The incorporation of caged NAD(P)$^+$ into crystalline enzyme complexes demands placement of the nitrobenzyl group in a region of the cofactor molecule which eliminates biological activity without seriously compromising binding. The placement of the nitrobenzyl group at the carboxamide was encouraged by the negligible biological activities of NAD(P)$^+$ analogs with similiary placed bulky additions.[9] Furthermore, the carboxamide has generally been shown to be a marginal contibutor to cofactor binding when compared with the high binding contributions of the pyrophposphate and adenosine moieties.[10]

Scheme 1



Scheme 2[a]



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[a] Reaction conditions: (a) $MeONH_3Cl$, pyridine/ethanol, reflux; (b) borane (1 M), THF, reflux; (c) nicotinoyl chloride hydrochloride, DMF, triethylamine.

Synthesis of caged NAD(P)$^+$ was carried out by a combination of enzymatic and synthetic organic methods. Incorporation of the nitrobenzyl group into the NAD(P)$^+$ cofactors was achieved by the use of NADase glycohydrolase from porcine brain, for the stereoselective exchange of the nicotinamide goup of NAD$^+$ or NADP$^+$ with *N*-(2-nitrobenzyl)nicotinamide (**1**) (Scheme 1).[11] Pig brain NADase has shown a tolerance for the exchange of a wide spectrum of substituted nicotinamides and circumvents the use of involved synthetic routes.[12]

Synthesis of *N*-(2-nitrobenzyl)nicotinamide was carried out through the coupling of 2-nitrobenzylamine with nicotinoyl chloride (Scheme 2). 2-Nitrobenzylamine was synthesized by the conversion of *o*-nitrobenzaldehyde to the oxime ether followed by borane reduction to the amine in 60% yield.[13,14]

Incoproration of **1** into NAD$^+$ and NADP$^+$ proceeded by the reaction of an excess of **1** and NADase for reaction times of 4 and 7.5 h, respectively. Yields of 10% for caged NAD$^+$ and 20% for caged NADP$^+$ (**2**) were obtained following purification.[15] Neither yield was optimized although the modest water solubility of **1** limits the use of an excess of **1** to drive the exchange further to completion. Characterization of caged NAD(P)$^+$ was accomplished by electrospray mass spectrometry, ^{1}H NMR, UV spectroscopy, and HPLC.[16] The purity of **2** was determined to be 95% by HPLC analysis and the purity of caged NAD$^+$ to be 97%.

The photorelease of NADP$^+$ was verified under aqueous conditions by ^{1}H NMR, HPLC, electrospray mass spectrometry,

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(14) This route to *o*-nitrobenzylamines has also proved an efficent means of converting *o*-nitroacetophenone and 2,6-dinitrobenzaldehyde to the corresponding amines in 50 and 60% yields, respectively.

(15) Exchange of the nicotinamide group of NAD(P)$^+$ with water by NADase to form the corresponding ADPR or ATPR products is the dominant pathway under conditions of low *N*-(2-nitrobenzyl)nicotinamide concentration.

(16) See Supporting Information for detailed analysis.

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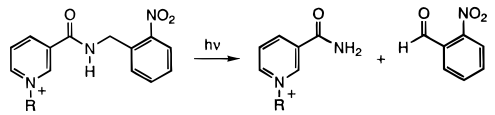
Scheme 3



ultraviolet spectroscopy, and biological assay.[16] Compound **2** was photolyzed by exposure to an excimer laser at 308 nm (Scheme 3). The yield of $NADP^+$ measured after complete disappearance of **2** was 40 ± 2.5% over the pH range of 6.2−8.0.[17] This relatively low final yield of conversion to $NADP^+$ was considered as being related to the nitroso aldehyde byproduct. The *o*-nitroso aldehyde has been demonstrated to be biologically reactive.[18] The use of thiols to form less reactive adducts with nitroso aldehydes has been applied to increase yields and minimize adverse biological effects.[2a] Additon of dithioerythritol (DTE) to photolysis experiments, however, results in only a modest increase of 50% $NADP^+$ released.[19] Several minor byproducts could be observed using HPLC analysis. The limited response to DTE addition may have a conformational origin. The solution conformation of $NADP^+$ places the nicotinamide and adenine groups in an equilibrium between open and stacked forms.[20] Both forms are known to exist in appreciable amounts at 25 °C and possess distinctive chemistries with regard to addition by nucleophiles.[20] The stacked form places the *o*-nitrobenzyl group in close proximity to both the adenine N-1 and N-6, which have been shown to be reactive with a variety of alkylating agents.[21] The 1-(2-nitrophenyl)ethyl group has been used as a caging group to enhance yields of release by virture of its less reactive *o*-nitrosoacetophenone byproduct.[2a] Incorporation of similar groups into $NAD(P)^+$ is currently being pursued.

The quantum efficency of $NADP^+$ photorelease was measured at 308 nmn using excimer laser photolysis. Potassium ferrioxolate actinometry was used to measure laser intensity and HPLC to assess the amount of $NADP^+$ released.[22] A value of $\Phi_{app} = 0.19 \pm 0.02$ for the appearance of NAD was obtained and $\Phi_{dis} = 0.30 \pm 0.02$ for the disappearence of **2**.[16]

The biological inactivity of caged $NADP^+$ was established for several $NADP^+$ dependent dehydrogenases.[16] Inactivity relative to controls under conditions of high enzyme activity was demonstrated for glucose 6-phosphate dehydrogenase, aldehyde dehydrogenase, ethanol dehydrogenase, and aromatic alcohol dehydrogenase. The absence of enzymatic reduction of caged $NADP^+$ was established by monitoring for absorption increases in the 300−400 nm range corresponding to the 1,4-dihydropyridine chromophore ($\lambda_{max} = 335$ nm, $\epsilon = 6220$) of NADPH. The biological activity of photoreleased $NADP^+$ was ascertained by the addition of aldehyde dehydrogenase to completely photolyzed samples of **2**. The new absorption maximum at 335 nm produced upon addition of aldehyde dehydrogenase corresponds to the enzymatic reduction of photoreleased $NADP^+$ to NADPH.[16] Values of 55−60% $NADP^+$ released are measured by this method. The photolysis of **2** was also carried out in the presence of ethanol dehydrogenase, demonstrating reduction of released $NADP^+$ commensurate with exposure to ultraviolet irradiation.[16]

In summary, the incorporation of the *o*-nitrobenzyl group into the $NAD(P)^+$ cofactors has been demonstrated. The biological inactivtiy of caged $NADP^+$ has been verified as well the release of $NADP^+$ upon excimer laser irradiation and quantum efficencies of release. The incorporation of other photolabile groups into $NAD(P)^+$ is currently being investigated.

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Supporting Information Available: Experimental procedures and spectra (34 pages). See any current masthead page for ordering and Internet instructions.

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(17) Conditions: 0.5 mL of 0.1 mM **2** in aqueous solution with appropriate buffer (pH = 6.0−7.1, 150 mM ammonium formate; pH = 7.1−8.0, 75 mM Tris) was placed in a 1 cm quartz cuvette with magnetic stirrer at 22 °C. Photolysis carried out under standard atmospheric conditions as net yields of conversion demonstrated no oxygen dependence. The samples were uniformly irradiated with 120 pulses from an excimer laser (308 nm, 8 mJ/pulse). $NADP^+$ present in photolyzed samples was measured by HPLC using a YMC C_{18} analytical column. Amounts of $NADP^+$ released were quantified by using an HPLC standard of $NADP^+$ (99%, Sigma, ϵ = 18 000, 260 nm) to correlate HPLC peak integrations to moles of $NADP^+$ released. See Section 2b of the Supporting Information for details of HPLC conditions.

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(19) Conditions: 0.5 mL of 0.1 mM **2** in aqueous solution with 150 mM ammonium formate (pH = 7.1, 22 °C) was placed in a 1 cm quartz cuvette with magnetic stirrer. DTE concentrations ranged from 0.2−25 mM. Solutions were degassed with argon. The samples were uniformly irradiated with 120 pulses from an excimer laser (308 nm, 8 mJ/pulse). $NADP^+$ present in photolyzed samples was measured by the methods described in ref 17. Maximum yield of 50% obtained for concentrations between 0.5 and 10 mM DTE.

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