Interactions between Transhydrogenase and Thio-nicotinamide Analogues of NAD(H) and NADP(H) Underline the Importance of Nucleotide Conformational Changes in Coupling to Proton Translocation*

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Transhydrogenase couples the reduction of NADP+ by NADH to inward proton translocation across mitochondrial and bacterial membranes. The coupling reactions occur within the protein by long distance conformational changes. In intact transhydrogenase and in complexes formed from the isolated, nucleotide-binding components, thio-NADP(H) is a good analogue for NADP(H), but thio-NAD(H) is a poor analogue for NAD(H). Crystal structures of the nucleotide-binding components show that the twists of the 3-carbothiamide groups of thio-NADP+ and of thio-NAD+ (relative to the planes of the pyridine rings), which are defined by the dihedral, $X_{\text{am}}$, are altered relative to the twists of the 3-carboxamide groups of the physiological nucleotides. The finding that thio-NADP+ is a good substrate despite an increased $X_{\text{am}}$ value shows that approach of the NADH prior to hydride transfer is not obstructed by the S atom in the analogue. That thio-NAD(H) is a poor substrate appears to be the result of failure in the conformational change that establishes the ground state for hydride transfer. This might be a consequence of restricted rotation of the 3-carbothiamide group during the conformational change.

Transhydrogenase is found in the inner membrane of animal mitochondria and in the cytoplasmic membrane of bacteria. The enzyme provides NADPH for biosynthesis and for reduction of glutathione, and in some mammalian tissues, it probably participates in the regulation of flux through the tricarboxylic acid cycle (1, 2). Under most physiological conditions transhydrogenase is driven in the “forward” direction by the proton electrochemical gradient ($\Deltap$) generated by respiratory (or photosynthetic) electron transport.

$\text{NADH} + \text{NADP}^+ + \text{H}_\text{out} \leftrightarrow \text{NAD}^+ + \text{NADPH} + \text{H}_\text{in}$  
(Eq. 1)

There is general agreement that coupling between the redox reaction and proton translocation is mediated by changes in protein conformation, although the character of these conformational changes is not known (reviewed in Refs. 3–5). Coupling mechanisms that involve large conformational changes operating over considerable distances are emerging as a common feature in proteins that translocate solutes/ions across membranes, and the amenable properties of transhydrogenase make it an attractive model in the search for fundamental principles. The enzyme has three components. The dI component, which binds NAD+ and NADH, and the dIII component, which binds NADP+ and NADPH, are extrinsic proteins protruding from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria), and dII spans the membrane. The enzyme is essentially a “dimer” of two dII-dIII “monomers,” although the polypeptide composition is variable among species. Crystal structures of Rhodospirillum rubrum di (6, 7), bovine dIII (8), human dIII (9, 10), and R. rubrum dI-dIII, complex (11, 12), and an NMR structure of R. rubrum dIII (13) have recently been published. Studies on the transient state kinetics of transhydrogenation reveal that the redox reaction between the two nucleotides is direct (14, 15). Thus, the nicotinamide and dihydrodinoctaminamide groups are brought into apposition to allow transfer of a hydride ion equivalent between the C-4 positions of the rings. The reaction is stereo-specific for the pro-R (A-side) of NAD(H) and the pro-S (B-side) of NADP(H) (16, 17).

Recent interpretations of kinetic and structural work on transhydrogenase have focused on the importance of conformational changes in the nucleotides as well as in the protein (3). It may be possible to test these interpretations through experiments using nucleotide analogues. Thio-NAD(H) and thio-NADP(H), which have a 3-carbothiamide substituent in place of the 3-carboxamide of the pyridine/dihydropyridine ring, have been used extensively in the study of soluble “dehydrogenases” (18). The binding properties of the analogues can be different relative to those of the physiological nucleotides and the catalytic rate can be affected; both increases and decreases have been observed in different enzymes (19). An x-ray structure of dihydrofolate reductase (DHFR)1 with bound thio-NAD+ showed how small distortions of the nucleotide conformation can lead to pronounced effects on catalysis (20).

Because the absorbance band of its reduced form is red-shifted relative to that of the physiological substrate (and therefore has minimal spectral overlap with NADH), thio-NADP+ has often been used to monitor the activity of proton-

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1 The abbreviations used are: DHFR, dihydrofolate reductase; thio-NADP+, 3-carbothiamide derivative of NADP+; AcPdAD, 3-acetylpyridine adenine dinucleotide; Mops, 4-morpholinepropanesulfonic acid.

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translocating transhydrogenase (21), but few experiments have been carried out using thio-nicotinamide analogues with a view to elucidating mechanistic details of the enzyme. In this report we compare hydride transfer rates to thio-NADP⁺ and to thio-NADP⁺ with those to NADP⁺ and NADP⁺, respectively, in both the intact R. rubrum transhydrogenase and in dIII/dIII₁ complexes. It emerges that thio-NADP⁺ is a poor substrate in the dI site, but thio-NADP⁺ is a good substrate in the dIII site. To attempt to explain these differences, we have solved the crystal structures of human dIII in its thio-NADP⁺ form (for comparison with dIII.NADP⁺; Protein Data Base 1Djl) and of the R. rubrum dIII₁dIII₂ complex loaded with thio-NADP⁺ and NADP⁺ (for comparison with complex loaded with NADP⁺ and NADP⁺; Protein Data Bank 1HZZ). The increased van der Waals’ radius of the S atom in the carboxamide group (1.9 Å compared with 1.4 Å of the O atom) and the increased length of the C = S bond (1.65 Å compared with 1.25 for C = O) have only quite subtle effects on the conformation of the bound nucleotides and the arrangement of the side chains of invariant amino acids at the binding site. We explain the results in terms of the structural changes at the catalytic center that are required to bring together the nicotinamide and dihydronicotinamide rings during hydride transfer, and we discuss the conclusions in the context of the suggestion that these structural changes are associated with proton translocation.

EXPERIMENTAL PROCEDURES

Recombinant dI and dIII (wild type and the E155W mutant) from R. rubrum transhydrogenase and human heart dIII were expressed in Escherichia coli and purified by column chromatography as described in Refs. 22–25. After supplementing with 25% glycerol, they were stored at –20 °C. Thawed proteins were either used directly in experiments or were first concentrated in Vivaspin centrifugal filters (5-kDa cut-off for dIII and 10-kDa cut-off for dI). Protein concentrations (given with respect to subunits) were determined by the microtannin procedure (26). Complexes (dIIIdIII₁) of dI and dIII are generated spontaneously (Kₚ < 60 mM (27)) upon mixing the two components in solution.

The dIII proteins are normally isolated in their NADP⁺-bound forms. Where required, the NADP⁺ was replaced by NADPH as described in Ref. 15. To replace with thio-NADP⁺, human dIII and R. rubrum dIIIdIII₁ were first washed in Vivaspin filters with 10 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 4 μM NADP⁺. The protein (~35 mg ml⁻¹) was then incubated in 10 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 10 mM thio-NADP⁺ at 4 °C for 1 h, a period sufficient to permit release of all tightly bound NADP⁺ (24). In crystallization experiments and in measurements of the cyclic reaction (see below), this solution was used directly. In stopped flow experiments and in measurements of nucleotide release, the solution was washed again in 10 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, and 50 μM thio-NADP⁺.

Everted cytoplasmic membranes (chromatophores) were isolated from phototrophically grown cultures of wild-type R. rubrum strain S1 and from a transhydrogenase-overexpressing strain RTB2 (28) by French pressing the cells as described in Ref. 29. The bacteriochlorophyll concentration was determined using the in vivo extinction coefficients of 140 μm⁻¹ cm⁻¹ at 880 nm (30). Where indicated, the dl component was washed from the membranes by centrifugation in the absence of NADP(H) (22). Reconstitution with recombinant dl protein was achieved by simple mixing.

Assays of steady state transhydrogenation were performed at 25 °C on a Perkin Elmer Lambda 16 double-beam spectrophotometer using 100 mM potassium phosphate (pH 7.0) as buffer and 200 μM NAD(P)H (final concentration). Absorbance changes at 340 nm (NAD(P)H) and 265 nm (NAD(P)) were recorded at 20°C using the programs MOLSCRIPT (38) and TURBO-FRODO (39). The wild-type structures of dIII with NADP⁺ bound and dIII₁dIII₂ with bound NADP⁺ were refined against the structure factor amplitudes of dIII with NADP⁺ bound and dIIIdIII₁ with NADP⁺ bound and NADP⁺ bound. The refinement statistics are summarized in Table I. The refinement was carried out using the programs SCALA (35). The wild-type structures of dIII with NADP⁺ bound and dIIIdIII₁ with NADP⁺ bound were refined against the structure factor amplitudes of dIII with thio-NADP⁺ bound and dIIIdIII₁ with NADP⁺ bound, respectively, using the program CNS (36, 37). The refinement statistics are summarized in Table I. Simulated annealing omit maps, both fₒ – f and 2fₒ – f, calculated using CNS, confirmed the calculated positions of the atoms in the carboxamide groups of the two structures. Further confirmation of the sulfur atom positions was obtained from test refinements using various combinations of the parameter files for the physiological and analogue nucleotides and the reflection files from the structures. The information for the sulfur atom positions was found to reside predominantly in the reflection files of the thio-NADP⁺ (9) files for both structures. The cut-off used for the hydrogen bond determination were 2.35–3.2 Å. Ribbon diagrams were prepared using the programs MOLSCRIPT (38) and TURBO-FRODO (39). The human dIII.thio-NADP⁺ structure and R. rubrum dl.dIII₁ with bound thio-NADP⁺ and NADP⁺ appear as Protein Data Bank entries 1PT9 and 1PTJ, respectively.
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Mixtures of isolated, purified dI and dIII of R. rubrum transhydrogenase spontaneously form stable dIdIII complexes (11, 22, 27). The steady state rates of oxidation of NADPH by NAD+, by thio-NAD+, and by AcPdAD+ catalyzed by dIdIII complexes were all very similar (~2 mol mol⁻¹ dm³ min⁻¹) but, even more than in the intact enzyme, these reverse transhydrogenations are limited by the very low rate of product NADP⁺ release (23). Cyclic reduction of thio-NAD⁺ by NADH plus NADPH catalyzed by dIdIII complexes (140 mol mol⁻¹ dm³ min⁻¹; Scheme 1A) was considerably slower than cyclic reduction of AcPdAD⁺ by NADH plus NADPH (typically 2000–3000 mol mol⁻¹ dm³ min⁻¹ (12, 23); Scheme 1B). Following the same arguments as above, this indicates that, as in the intact enzyme, thio-NAD⁺ is a very poor acceptor of hydride equivalents from NADPH.

Experiments in the stopped flow spectrophotometer provide complementary information. It was previously shown that mixing NADPH-loaded dI2dIII complexes with AcPdAD⁺ leads to a rapid burst of hydride transfer preceding the steady state reaction (15); the burst arises because the binding of AcPdAD⁺, hydride transfer, and release of AcPdADH are all very fast relative to the rate of NADP⁺ release. Subsequently, measurements of changes in Trp fluorescence revealed an equivalent rapid burst of reaction between NADPH-loaded dIdIII complexes and NAD⁺ (42, 43). In the experiment shown in Fig. 2, NADPH-loaded dIdIII complexes were mixed in the stopped flow spectrophotometer with thio-NAD⁺. A burst of reaction was observed but with a much slower rate than that observed with either NAD⁺ or AcPdAD⁺ as hydride acceptors. Approximately similar concentrations of AcPdAD⁺, NAD⁺, and thio-NAD⁺ were required to give the maximum rate constants for the respective reactions (kapp was ~550 s⁻¹ for NADPH → AcPdAD⁺, ~600 s⁻¹ for NADPH → NAD⁺, and ~8 s⁻¹ for NADPH → thio-NAD⁺). In a subsequent experiment, dIII loaded with NADPH from one syringe was mixed with dI plus thio-NAD⁺ (1.0 mM after mixing) from the other. Again the burst kinetics were observed and with a kapp = ~6 s⁻¹ (data not shown), proving that the slow rate of reaction is not a result of slow binding of thio-NAD⁺ to dI. The analysis of the kapp values in terms of their microscopic rate constants, for AcPdAD⁺ and NAD⁺ as hydride acceptors, was discussed previously (15).

The transient state kinetics of forward transhydrogenation on dI2dIII complexes with AcPdADH/NADP⁺ and with NADH/NAD⁺ were described in earlier works (31, 42, 43). These reactions also take place as a rapid single-turnover burst, here the slow steady state rate resulting from limiting NADPH release. For AcPdADH/NADP⁺, measured from the 375 nm absorbance change at saturating AcPdADH, kapp = ~90 s⁻¹, and for NADH/NAD⁺, measured from a Trp fluorescence change under continuous flow conditions at saturating NADH, kapp = ~21000 s⁻¹. When the dI2dIII complex loaded with NAD⁺ was mixed with thio-NADH in the stopped flow spectrophotometer, a single-turnover burst of reaction was observed, but it was considerably slower than with either AcPdADH or NADH as hydride donor (kapp = ~0.7 s⁻¹ at saturating thio-NADH).

Thio-NAD⁺ Is a Good Substrate in the Forward Reaction of R. rubrum Transhydrogenase—The use of thio-NAD⁺ as an analogue for NAD⁺ in the forward reaction (Equation 1) catalyzed by transhydrogenases from several sources is well documented (21). The steady state rate of the reaction catalyzed by wild-type R. rubrum chromatophores with saturating concentrations of nucleotides in dark conditions is typically 0.1 mol mol⁻¹ bacteriochlorophyll min⁻¹. With saturating photosynthetic illumination, the rate increases in the order of 10-fold as a result of the increased proton electrochemical gradient. Be-
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**Scheme 1.** The cyclic reaction of transhydrogenase. E represents an enzyme catalytic site at the interface between dI and dIII. The dIII nucleotide-binding site is shown to be permanently occupied by either NADP⁺/NADPH (A and B) or thio-NADP⁺/thio-NADPH (C). The two double-headed arrows in each panel show consecutive events at the catalytic site. For example, in A, at the left arrows, NADH binds (to dI) and reduces the (dIII-bound) NADP⁺, and NAD⁺ then dissociates; at the right arrows, thio-NAD⁺ binds (to dI) and oxidizes the (dIII-bound) NADPH, and thio-NADH then dissociates.

**Fig. 2.** Transient state kinetics of thio-NAD⁺ reduction by NADPH catalyzed by *R. rubrum* dI-dIII complexes. For the experiments shown in the main figure (A), the first syringe of the stopped flow spectrophotometer contained 50 μM dI and 25 μM dIII-NADPH in 20 mM Hepes, pH 8.0, 10 mM (NH₄)₂SO₄, and 1 mM dithiothreitol. The second contained varying concentrations of thio-NAD⁺ in the same buffer. The solutions were mixed in a 1:1 ratio; the absorbance change was measured from the change in dIII reduction (the latter measured from the change in dIII absorbance at 395 nm in a solution containing dI (to give the concentration shown in the figure) and dIII (30 nM) in 50 mM Mops, pH 7.2, 2 mM MgCl₂, 200 μM AcPdAD, 200 μM NADH, and 100 μM NADP⁺. Cyclic reduction of AcPdAD⁺ by NADPH via bound thio-NADP⁺ (see Experimental Procedures). The experiments were carried out under similar conditions, but the dIII protein was pre-treated to exchange its NAD⁺ for thio-NAD⁺ (see Experimental Procedures), and the NAD⁺ in the assay buffer was replaced with thio-NAD⁺. The temperature was 25 °C. The curves through the data points are not meant to imply a known or modeled relationship. Inset B shows an average of six traces for a typical experiment at a thio-NAD⁺ concentration of 2.0 mM. For comparison, inset C shows a typical transient state burst of the reduction of AcPdAD⁺ by NADPH under similar conditions (syringe 1 contained 100 μM dI and 50 μM dIII-NADPH, and syringe 2 contained 2 mM AcPdAD⁺; the absorbance change was measured at 375 nm and averaged over six traces) (15). The horizontal bar corresponds to 200 ms; the vertical bar is 0.004 ΔA in B and 0.001 ΔA in C. The temperature was 20 °C.

**Fig. 3.** Cyclic reduction of AcPdAD⁺ by NADH on dI-dIII complexes is supported by either NADP⁺ or thio-NADP⁺. Cyclic reduction of AcPdAD⁺ by NADH via bound NADP⁺ (see Experimental Procedures). With both hydride donors a rapid burst of thio-NADP⁺ formation preceded the very slow steady state reaction (Fig. 4). This points to a kinetic mechanism similar to that observed with other nucleotides: rapid binding of NADH (or AcPdADH), rapid hydride transfer, and slow release of thio-NADPH. At close to saturating concentrations of AcPdADH (100–200 μM) the dominant (and faster) kinetic component in the burst had a k_app value of ~200 s⁻¹, which compares with a k_app of ~90 s⁻¹ for AcPdADH → NADP⁺ (31). Equivalently, the increase in the k_app for the burst of thio-NAD⁺ reduction with the initial concentration of NADH was similar to that seen for the burst of NAD⁺ reduction (the latter measured from the change in dIII Trp fluorescence in the E155W mutant (43)). In neither set of experiments was there any indication of saturation by NADH substantially slower than the respective reactions with NADP⁺ and NADPH.

Second, the transient state kinetics of thio-NAD⁺ reduction by NADH and by AcPdADH on dI-dIII complexes were investigated in the stopped flow spectrophotometer. With both hydride donors a rapid burst of thio-NADP⁺ formation preceded the very slow steady state reaction (Fig. 4). This points to a kinetic mechanism similar to that observed with other nucleotides: rapid binding of NADH (or AcPdADH), rapid hydride transfer, and slow release of thio-NADPH. At close to saturating concentrations of AcPdADH (100–200 μM) the dominant (and faster) kinetic component in the burst had a k_app value of ~200 s⁻¹, which compares with a k_app of ~90 s⁻¹ for AcPdADH → NADP⁺ (31). Equivalently, the increase in the k_app for the burst of thio-NAD⁺ reduction with the initial concentration of NADH was similar to that seen for the burst of NAD⁺ reduction (the latter measured from the change in dIII Trp fluorescence in the E155W mutant (43)). In neither set of experiments was there any indication of saturation by NADH.
up to the limit of resolution of the instrument ($k_{app} = -800 \, s^{-1}$, reached at 100–200 $\mu$M nucleotide). The data show that the rates of hydride transfer from NADH to NADP$^+$ and to thio-NADP$^+$ are similar.

**Rate of Release of Thio-NADP$^+$ from the dIII Component of R. rubrum Transhydrogenase**—Isolated dIII is locked in an "occluded state" resembling an intermediate in turnover of the intact enzyme (3). An important property of the occluded state is its slow rate of exchange of bound NADP(H) with nucleotide in the solvent. We have therefore investigated the rate of thio-NADP$^+$ release from dIII. The E155W mutant of R. rubrum dIII has very similar kinetic and thermodynamic properties to wild-type dIII, but fluorescence from its unique Trp residue is sensitive to the redox state of bound nucleotide (24). Thus, the fluorescence emission from dIII.NADP$^+$ is 25% higher than that from dIII.NADPH, and this fact can be used to determine the occupancy of the binding site. In the present experiments, we observed that Trp fluorescence from dIII.thio-NADP$^+$ is even lower than that from dIII.NADPH and that the Trp fluorescence of dIII.thio.NADPH is about 10% lower than that of dIII.thio-NADP$^+$ (data not shown). The experiment illustrated by the upper trace in Fig. 5 was performed with dIII.E155W presaturated with thio-NADP$^+$. Following a short period of preincubation, during which the rates of thio-NADP$^+$ release and rebinding were equalized, NADPH was added to the protein solution. The initial, prompt fluorescence decrease was due to inner filtering by the nucleotide. The subsequent slow increase in Trp fluorescence was the result of replacement of the bound thio-NADP$^+$ by NADPH. Separately it was established that the NADPH used in the experiment was in excess, and under these conditions, the fluorescence increase gives the first order rate constant for thio-NADP$^+$ release (see scheme and compare with Refs. 27 and 44). The calculated value ($k_{off} = 0.028 \, s^{-1}$) is similar to that determined for NADP$^+$ release ($k_{off} = 0.022 \, s^{-1}$). In a complementary experiment (Fig. 5, lower trace), excess thio-NADP$^+$ was added to dIII.E155W in its NADP$^+$ form. Following the rapid, initial, inner filtering effect, the decrease in fluorescence is attributed to the replacement of the physiological nucleotide by the analogue. The rate constant for the fluorescence decrease corresponds to that for NADP$^+$ release; the calculated value ($k_{off} = 0.030 \, s^{-1}$) is indeed similar to that determined following an NADPH pulse (27).

**Perturbation in the NMR Spectrum of R. rubrum dIII by Thio-NADP$^+$ and Thio-NADPH**—In the HSQC experiment, the $^1$H and $^{15}$N spins of amide groups in a protein are correlated. Almost all the peaks in the $^1$H,$^{15}$N HSQC spectrum of

![Fig. 4. Transient state kinetics of thio-NADP$^+$ reduction by NADH and by AcPDADH catalyzed by R. rubrum dIIdIII, complexes. The first syringe of the stopped flow spectrophotometer contained 50 $\mu$M dI and 25 $\mu$M dIII.thio-NADP$^+$ in 20 mM Hepes, pH 8.0, 10 mM (NH$_4$)$_2$SO$_4$, and 1 mM dithiothreitol. The second contained either 100 $\mu$M NADH (trace A) or 100 $\mu$M AcPDADH (trace B) in the same buffer. The solutions were mixed in a 1:1 ratio, and the absorbance followed at 395 nm. The temperature was 20 °C.](image-url)

$R.\, rubrum$ $NADH$ and by AcPdADH catalyzed by the $1H$ and $15N$ spins of amide groups in a protein are correlated. Almost all the peaks in the $1H,15N$ HSQC spectrum of $dIII.NADP$ sensitive to the redox state of bound nucleotide (24). Thus, the wild-type $dIII$, but fluorescence from its unique Trp residue is the occupancy of the binding site. In the present experiments, that from $dIII.NADPH$, and this fact can be used to determine release; the calculated value ($k_{off} = 0.022 \, s^{-1}$, excess thio-NADP$^+$ in 20 mM Hepes, pH 8.0, 10 mM (NH$_4$)$_2$SO$_4$, and 1 mM dithiothreitol. The second contained either 100 $\mu$M NADH (trace A) or 100 $\mu$M AcPDADH (trace B) in the same buffer. The solutions were mixed in a 1:1 ratio, and the absorbance followed at 395 nm. The temperature was 20 °C. The initial, prompt fluorescence decrease was due to inner filtering by the nucleotide. The subsequent slow increase in Trp fluorescence was the result of replacement of the bound thio-NADP$^+$ by NADPH. Separately it was established that the NADPH used in the experiment was in excess, and under these conditions, the fluorescence increase gives the first order rate constant for thio-NADP$^+$ release (see scheme and compare with Refs. 27 and 44). The calculated value ($k_{off} = 0.028 \, s^{-1}$) is similar to that determined for NADP$^+$ release ($k_{off} = 0.022 \, s^{-1}$). In a complementary experiment (Fig. 5, lower trace), excess thio-NADP$^+$ was added to dIII.E155W in its NADP$^+$ form. Following the rapid, initial, inner filtering effect, the decrease in fluorescence is attributed to the replacement of the physiological nucleotide by the analogue. The rate constant for the fluorescence decrease corresponds to that for NADP$^+$ release; the calculated value ($k_{off} = 0.030 \, s^{-1}$) is indeed similar to that determined following an NADPH pulse (27).

**Perturbation in the NMR Spectrum of R. rubrum dIII by Thio-NADP$^+$ and Thio-NADPH**—In the HSQC experiment, the $^1$H and $^{15}$N spins of amide groups in a protein are correlated. Almost all the peaks in the $^1$H,$^{15}$N HSQC spectrum of $R.\, rubrum$ $dIII.NADP^+$ are now assigned (13). The HSQC spectrum of dIII.thio-NADP$^+$ differed from that of dIII.NADP$^+$ only in amides that are spatially close to the nicotinamide ring of the nucleotide (Table II), especially those in the "nicotinamide binding loop" between strand $\beta$2 and helix B. This indicates that the only changes in the protein structure caused by substituting the physiological nucleotide with the analogue are in the locality of the thio-nicotinamide ring.

Chemical shift changes are observed in the HSQC spectra of $R.\, rubrum$ (33) and $E.\, coli$ dIII (45) when bound NADP$^+$ is replaced by NADPH. The changes, mapped onto the high resolution structures of the $R.\, rubrum$ protein, reveal that magnetic perturbations (atomic displacements or charge redistributions or both (46)) not only take place in the vicinity of the nicotinamide ring but also extend into helix D/loop D and loop E of the protein, and they might reflect events associated with the gating mechanism of transhydrogenase (10). Chemical shift changes in the $^1$H,$^{15}$N HSQC spectrum of $^{15}$N-labeled dIII, consequent upon the reduction of bound thio-NADP$^+$ by addition of a low concentration of unlabelled $R.\, rubrum$ dI protein plus NADH, are listed in Table III. Reduction was not complete (−30%), but clearly, the amino acid residues whose amide groups are affected are the same as those affected by substitution of NADP$^+$ by NADPH (33).

**Crystal Structures of Isolated Transhydrogenase Components with Bound Thio-analogues of Nicotinamide Nucleotides**—To try to understand why thio-NAD$^+$ is a poor substrate for transhydrogenase but thio-NADP$^+$ is a good substrate, we have determined high resolution structures of isolated components of the enzyme in different nucleotide-bound states. Human dIII in its thio-NAD$^+$ form was crystallized under conditions similar to those described for the protein in its NAD$^+$ form (9, 10), and its structure was solved by x-ray diffraction. The fold of dIII.thio-NAD$^+$ is very similar to that of dIII.NADP$^+$ (root mean square difference of $C_\alpha = 0.3 \, \AA$). Briefly, the protein adopts a Rossmann fold; it is a six stranded parallel $\beta$ sheet flanked by helices, organized into two $\beta\alpha\beta\alpha$ motifs. As in the classical Rossmann fold, the NADP$^+$/thio-NADP$^+$ is bound in a crevice at the C-terminal end of the $\beta$ sheet, but the orientation of the nucleotide is reversed relative to that found in other structures; the adenine moiety is located over the second $\beta\alpha\beta\alpha$ motif, and the nicotinamide mononucleotide is located over the first. The nicotinamide and thio-nicotinamide rings in the respective structures are bound by the loop between strand $\beta$2 and helix B (Fig. 6). A striking difference between the two structures is that, in dIII.NADP$^+$, the 3-carboxamide group is approximately coplanar with the pyridine ring, the oxygen atom being trans to C-2 ($X_{am} = 179^\circ$), but in dIII.thio-NADP$^+$, the 3-carboxamidic is twisted relative to the pyridine plane ($X_{am} = 140^\circ$). However, the hydrogen bond between N-7 of the thio-nicotinamide and the Ala925 carbonyl group is preserved, and the pyridine ring of the thio-NADP$^+$ is still maintained in a similar position in the binding loop to that of the NADP$^+$ pyridine ring; contacts between the ring atoms and side chains of amino acid residues in the loop move only slightly. Importantly, the C-4 atom moves no more than 0.3 Å from the polypeptide backbone, and the si face of the ring is exposed to the solvent in the same way as the physiological nucleotide. It will be discussed below that during hydride transfer the si face of the NADP$^+$ (thio-nicotinamide at C-4 is presented to the pro-R hydrogen at the C-4 atom of the NADH dihydronicotinamide. The confinement of structural changes to the vicinity of the nicotinamide ring in the crystal-line state is nicely consistent with the limited changes in the amide chemical shifts observed in solution experiments by NMR (Table II).
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Fig. 5. Slow release of thio-NADP\(^+\) and NADP\(^+\) from isolated R. rubrum dIII. Upper trace, the protein (dIII.E155W of R. rubrum transhydrogenase) was pretreated to exchange its NADP\(^+\) for thio-NADP\(^+\) (see “Experimental Procedures”). A sample of this was suspended in 20 mM Mops, pH 7.2, 10 mM KCl, 4 mM MgCl\(_2\) to give a concentration of 1 μM NADPH was added where shown to give a final concentration of 50 μM. Lower trace, dIII.E155W in its NADP\(^+\) form was suspended in the same buffer again to give 1 μM. Thio-NADP\(^+\) was added where shown to give a final concentration of 50 μM. An upward deflection represents a fluorescence increase. The boxed schemes show the models for nucleotide dissociation and rebinding (see text and Refs. 24 and 44). The temperature was 25°C.

| TABLE II | Chemical shift perturbations in backbone amide groups of R. rubrum dIII upon substituting NADP\(^+\) with thio-NADP\(^+\) |
|-----------|---------------------------------------------------------------|
| Residue   | Chemical shift perturbation of backbone amide (Hz)            |
| Gly\(^{54}\) | 36.6                                                          |
| Val\(^{87}\) | 73.9                                                          |
| Ala\(^{86}\) | 161.0                                                         |
| Gly\(^{99}\) | 40.6                                                          |
| Arg\(^{80}\) | 49.6                                                          |
| Met\(^{91}\) | 56.0                                                          |
| Val\(^{77}\) | 77.4                                                          |
| Leu\(^{98}\) | 39.7                                                          |
| Glu\(^{101}\) | 34.6                                                          |
| Leu\(^{102}\) | 48.8                                                          |
| Tyr\(^{117}\) | 48.0                                                          |

We have also determined the x-ray structure of the complex formed from a mixture of isolated dI and dIII components of R. rubrum transhydrogenase (compare Ref. 11), but in crystals grown in the presence of a combination of thio-NAD\(^+\) and NADP\(^+\), two oxidized nucleotides, ensure that hydride transfer does not take place during crystallization. Again the overall fold was not affected by the substitution of NAD\(^+\) by thio-NAD\(^+\) (root mean square difference of C\(_\text{α}\) = 0.3 Å). The complexes are dI.dIII\(_1\) heterotrimers. The two symmetrically organized dI polypeptides (A and B) are each composed of two domains (d.I.1 and d.I.2) that are separated by deep clefts and linked by two long helices. Both d.I.1 and d.I.2 comprise mostly parallel β-sheets flanked by helices and have the form and connectivity of the Rossmann fold. Only the cleft of d(I)B is associated with a dIII polypeptide. The fold of the dIII polypeptide and the conformation of its bound NADP\(^+\) are very similar to those seen in the structures of the isolated dIII.NADP\(^+\) of

![Diagram of the transhydrogenase structure](image-url)

| TABLE III | Chemical shift perturbations in backbone amide groups of R. rubrum dIII upon reducing bound NADP\(^+\) and bound thio-NADP\(^+\) |
|-----------|---------------------------------------------------------------|
| Residue   | Chemical shift perturbation of backbone amide (Hz) |
| Gly\(^{54}\) | 70.1                                                          |
| Tyr\(^{55}\) | 235.6                                                         |
| Ala\(^{86}\) | 258.4                                                         |
| Gly\(^{89}\) | 222.1                                                         |
| Arg\(^{90}\) | 155.4                                                         |
| Met\(^{91}\) | 59.4                                                          |
| Met\(^{95}\) | 51.1                                                          |
| Val\(^{97}\) | 79.6                                                          |
| Leu\(^{99}\) | 44.1                                                          |
| Asn\(^{116}\) | 50.1                                                          |
| Asp\(^{131}\) | 133.0                                                         |
| Asp\(^{132}\) | 99.5                                                          |
| Val\(^{133}\) | 123.7                                                         |
| Asn\(^{135}\) | 143.3                                                         |
| Lys\(^{139}\) | 349.4                                                         |
| Thr\(^{140}\) | 167.7                                                         |
| Asp\(^{141}\) | 219.7                                                         |
| Ser\(^{143}\) | 181.8                                                         |
| Ser\(^{144}\) | 139.2                                                         |
| His\(^{146}\) | 345.9                                                         |
| Gly\(^{149}\) | 50.6                                                          |
| Met\(^{149}\) | 224.5                                                         |
| His\(^{151}\) | 141.0                                                         |
| Val\(^{154}\) | 57.4                                                          |
| Lys\(^{156}\) | 48.7                                                          |
| Gly\(^{170}\) | 83.6                                                          |
| Tyr\(^{171}\) | 167.2                                                         |
| Ala\(^{172}\) | 133.9                                                         |
| Gly\(^{173}\) | 64.6                                                          |
| Val\(^{174}\) | 54.9                                                          |
| Glu\(^{177}\) | 70.0                                                          |
| Leu\(^{178}\) | 56.8                                                          |

Chemical shift perturbation of backbone amide groups (Hz) of R. rubrum dIII-NADPH, dIII-thio-NADPH, and dIII-thio-NADP\(^+\) were recorded as described under “Experimental Procedures” and “Results.” The chemical shift perturbation (see Table II) was measured for (NADP\(^+\) – NADPH) and for (thio-NADP\(^+\) – thio-NADPH) (the latter are described as tNADP\(^+\) and tNADPH in the table). Only residues with perturbations >50 Hz either for NADP\(^+\) – NADPH or tNADP\(^+\) – tNADPH are listed. Data for (NADP\(^+\) – NADPH) are similar to those presented previously (33) but include additional assignments in dIII.NADPH. NA, not assigned.
mammalian enzymes (see above). As observed in the *R. rubrum* dI2dIII1 complex crystallized with NAD+/H and NADP+/H and discussed in Refs. 27, 33, and 42, there is good electron density for (thio-)NAD+/H only in the A polypeptide of the new structure; the binding site is located at the C-terminal ends of the strands in the β-sheet of dI.2. The adenosine moieties of the NAD+/H and thio-NAD+/H bind in the same way. The nicotinamide and thio-nicotinamide rings occupy quite similar positions in the binding pocket, but there are differences of detail (Fig. 7). Most obviously, the dihedral angles, $X_{am}$, signifying the twist of the 3-carboxamide group relative to the pyridine ring, and $X_n$, the rotation of the pyridine ring relative to the nicotinamide ribose, are both altered in the dI(A)-bound thio-NAD+. Note that the orientation of the 3-carboxamide group of NAD+ in our earlier structure is unusual with the oxygen atom approximately cis to C-2 of the pyridine ring ($X_{am} = -180^\circ$). However, when these two proteins are loaded with thio-NAD+, the carbothioamide group of the analogue in both cases is twisted relative to the plane of the pyridine ring, although the twist has the opposite sense: $X_{am}$ is $-160^\circ$ in DHFR and $+140^\circ$ in dIII. The increased twist of the carbothioamide group in DHFR was suggested to avoid unfavorable contact between the sulfur atom and the CH group at position 4 of the pyridine ring (20). It was noted to be less than in a model compound, 2-methyl-4(thiocarbamoyl)-pyridine (47). McTigue et al. (20) concluded that a larger rotation in DHFR is prohibited by the binding site geometry of the protein and that the unfavorable interactions between the CH group of pyridine ring and the S atom are not completely relieved. The oppositely directed twist of the carbothioamide group in dIII (Fig. 6) might similarly result from this kind of structural compromise; the tendency of the group to twist from a coplanar position to prevent unfavorable contact between the large S atom and C4H is limited.

**DISCUSSION**

It is instructive to compare the crystal structures of DHFR and dIII from transhydrogenase. In both DHFR.NAD+ and human dIII.NAD+ the carboxamide group of bound nucleotide is in the trans position, approximately coplanar with the pyridine ring ($X_{am} = -180^\circ$). However, when these two proteins are loaded with thio-NAD+, the carbothioamide group of the analogue in both cases is twisted relative to the plane of the pyridine ring, although the twist has the opposite sense: $X_{am}$ is $-160^\circ$ in DHFR and $+140^\circ$ in dIII. The increased twist of the carbothioamide group in DHFR was suggested to avoid unfavorable contact between the sulfur atom and the CH group at position 4 of the pyridine ring (20). It was noted to be less than in a model compound, 2-methyl-4(thiocarbamoyl)-pyridine (47). McTigue et al. (20) concluded that a larger rotation in DHFR is prohibited by the binding site geometry of the protein and that the unfavorable interactions between the CH group of pyridine ring and the S atom are not completely relieved. The oppositely directed twist of the carbothioamide group in dIII (Fig. 6) might similarly result from this kind of structural compromise; the tendency of the group to twist from a coplanar position to prevent unfavorable contact between the large S atom and C4H is limited.
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by the constraints imposed by interactions between the nucleotide and the protein-binding pocket.

The constraints in the nucleotide-binding site in dI/(A) resulting from the substitution of NAD+ with thio-NAD+ in the R. rubrum dl,dIII complex have a different character. They appear to be dominated by unfavorable interactions between the bulky carbothiamide S atom and the protein-binding pocket, especially the side chain of Gln132. The carbothiamide group is twisted away from Gln132 into the plane of the pyridine ring, evidently incurring the penalty of a close contact between the S atom and C2H. The torsion angle of the glycosidic bond is distorted, the Gln132 side chain is displaced, there is enforced close contact (2.8 Å) between the carbothiamide-S atom and the Arg127 carboxyl, and the single hydrogen bond that links the carbothiamide group of NAD+ with Ile128 is broken (Fig. 7).

In the crystal structure of a ternary complex of DHFR, the rotated sulfur atom in the thio-NADP+ results in destabilization of the bound bioterror; the latter is moved away from the nicotinamide ring, and its temperature factors are increased relative to those in the NADP+ structure (20). This suggested an explanation for the finding that thio-NADPH is a very poor coenzyme for DHFR; in the formation of the transition state for hydride transfer, the mutual approach of the nicotinamide ring and the substrate pteridine ring are impeded by the sulfur atom of the thioamide. In contrast to DHFR, thio-NADP+ is a good substrate for intact transhydrogenase and its isolated components (1). The rapid rate of the cyclic reaction in R. rubrum dl,dIII complexes and of the single turnover burst of thio-NADP+ reduction by NADH and by AcPdADH show that the hydride transfer step is functioning normally (2). The reduction of thio-NADP+ by NADH in suspensions of membranes was stimulated upon generation of a proton electrochemical gradient by photosynthetic electron transfer, showing that the reaction is well coupled to proton translocation (3). The rates of release of NADP+ and thio-NADP+ from isolated dIII, thought to be locked in the occluded state, were very similar (4). The chemical shift changes in isolated dIII accompanying the reduction of thio-NADP+ are very similar to those resulting from NADP+ reduction; the change in magnetization through helix D/loop D and loop E, thought to be related to a gating step in the enzyme, is not substantially affected. It appears that neither the increased atomic radius of the sulfur atom nor the increased twist of the carbothiamide group compromise the behavior of the nucleotide in transhydrogenation. Whether hydride transfer occurs entirely by way of an over-the-barrier mechanism or whether there is a contribution from quantum mechanical tunneling is not clear (15), but it is evident that the C-4 atoms of the dihydropyridine and pyridine ring systems (for example, of NADH and NADP+) must approach one another to facilitate the reaction, and we conclude that the bulky sulfur atom of the analogue despite its rotated position does not prevent this approach.

The explanation as to why, in contrast, thio-NAD(H) is a poor substrate for transhydrogenase (in both the intact enzyme and in dl,dIII complexes) is more difficult to define, but driving force effects can probably be ruled out. There are differences in the standard redox potentials of the nucleotides used in this work, and these differences will lead to differences in the driving force at the hydride transfer step; in aqueous solution the E° values of thio-NAD+/thio-NADH, AcPdAD+/AcPdADH, and NAD+/NADH are −0.285, −0.247, and −0.320 volt, respectively (18) (the presence of a 2′-phosphate group on the adenine ribose does not significantly affect E°). However, pairs of experiments with the dl,dIII complex under comparable, single-turnover conditions show that these differences do not account for the observation that thio-NAD+ is a poor hydride acceptor, and thio-NADH is a poor hydride donor. Thus, in reverse transhydrogenation, the driving force on the reaction NADPH → thio-NAD+ is greater than that on NADPH → NAD+ (calculated from the solution E° values), but the rate of reaction is much slower. Equivalently, in forward transhydrogenation, the driving force on thio-NADH → NADP+ is greater than that on AcPdADH → NADP+, but again the rate of the reaction is much slower.

To understand the behavior of nucleotide in the dI site of transhydrogenase, it has to be recognized that there are probably changes in protein and nucleotide conformation in this site that precede and follow the hydride transfer reaction (3, 11, 12). The conformation of the A polypeptide and its bound nucleotide in the dl,dIII complex (Fig. 7) probably corresponds to that in an “open state” of the intact enzyme. This is the state in which nucleotide reactants bind and products dissociate during turnover, but it is important that hydride transfer is prevented in this state. After NADH and NADP+ binding (e.g. during forward transhydrogenation), the conformation is driven by protonation/deprotonation reactions associated with proton translocation into an occluded state in which hydride transfer does proceed. We suggest that the low reactivity of thio-NAD(H) results from an obstruction of the conformational changes occurring during interconversion of the open and occluded states. When NAD(H) from the A polypeptide of the dl,dIII complex is modeled into the B polypeptide to anticipate the pretransition state for hydride transfer to NADP+ in dIII, the dihydro- and nicotinamide rings are in a “distal” conformation; the C-4 atoms of the nucleotides are too far apart to allow hydride transfer (11). A switch to a “proximal” nucleotide position is required to bring the C-4 atoms into apposition in the distal state of NAD(H). A preference for the cis conformation of NADH is approximately 5 × 105 (and related nucleotides in the Cambridge Structural Database) the oxygen atom of the 3-carboxamido group is cis to C-2 of the nicotinamide ring (X^N = +0°). A preference for the cis conformation of this group is also revealed in theoretical studies (49–51). However, in the majority of protein crystal structures that have bound nicotinamide nucleotides, the oxygen is approximately trans to C-2 (X^N = ±180°); the conclusion is usually based on the pattern of hydrogen bond donors and acceptors to the carboxamido group because few structures have been determined at a high enough resolution to show it directly. Of fifty unique NAD(P)(H)-binding proteins (not including transhydrogenase dl) deposited most recently in the Protein Data Bank, only three had nucleotide in the cis conformation; of these, two had an hydrogen bond organization that did not exclude the possibility of a trans conformation and the resolution of the other was probably not good enough to discriminate. The nucleotide in the dI site of transhydrogenase is one of the few clear exceptions to the rule. Thus, the probable hydrogen bond between the 3-carboxamido group of NAD+ and the carboxyl of Ile128 in the R. rubrum dl,dIII complex (11), and of NADH and the equivalent atom in the dl,NADH struc-
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Buckley, P. A., Jackson, J. B., Schneider, T., White, S. A., Rice, D. W., and Baker, P. J. (2000) Structure 8, 809–815.

Prasad, G. S., Wahlberg, M., Sridhar, V., Sundaresan, V., Yamaguchi, M., Hafey, Y., and Stout, C. D. (2003) Biochemistry 42, 12745–12754.

Prasad, G. S., Sridhar, V., Yamaguchi, M., Hafey, Y., and Stout, C. D. (1999) Nat. Struct. Biol. 6, 1126–1131.

Jackson, J. B., Peake, S. J., and White, S. A. (1999) FEBS Lett. 464, 1–8.

Weiss, S. A., Peake, S. J., Quinn, P. G., Cotton, N. P. J., and Jackson, J. B. (2000) Structure 8, 1–12.

Cotton, N. P. J., White, S. A., Peake, S. J., McSweeney, S., and Jackson, J. B. (2004) Structure 9, 165–170.

van Boel, G. L., Quirk, P., Cotton, N. P. J., and Jackson, J. B. (2003) Biochemistry 42, 1217–1226.

Jesch, M., Smith, R. K., Quinn, P. G., Cotton, N. P. J., and Jackson, J. B. (2000) J. Biol. Chem. 275, 27535–27538.

Venning, J. D., Grimley, R. L., Bizouarn, T., Cotton, N. P. J., and Jackson, J. B. (1997) J. Biol. Chem. 272, 41557–41563.

Venning, J. D., Bizouarn, T., Cotton, N. P. J., Quirk, P. G., and Jackson, J. B. (1998) Eur. J. Biochem. 257, 202–209.

Lee, C. P., Simard-Duquene, N., Ennert, L., and Hoberman, H. D. (1965) Biochim. Biophys. Acta 105, 397–409.

Fisher, R. R., and Guileory, R. J. (1971) J. Biol. Chem. 246, 4867–4893.

Wennerhaus, C., and Jekl, R. (1987) in Pyridine Nucleotide Coenzymes (Dolphin, D., Poulson, R., and Avramovic, O., eds) pp. 449–568, John Wiley & Sons, Inc., New York.

Cook, P. F., and Bertagnolli, B. L. (1987) in Pyridine Nucleotide Coenzymes (Dolphin, D., Poulson, R., and Avramovic, O., eds) pp. 455–468, John Wiley & Sons, Inc., New York.

McTigue, M. A., Davies, J. F., Kaufman, B. T., and Kraut, J. (1993) Biochemistry 32, 6855–6862.

Rydstrom, J. (1979) Methods Enzymol. 55, 261–275.

Diggle, C., Hutton, M., Jones, G. R., Thomas, C. M., and Jackson, J. B. (1995) Eur. J. Biochem. 228, 719–726.

Diggle, C., Bizouarn, T., Cotton, N. P. J., and Jackson, J. B. (1996) Eur. J. Biochem. 241, 162–170.

Peake, S. J., Venning, J. D., Cotton, N. P. J., and Jackson, J. B. (1999) Biochim. Biophys. Acta 1413, 81–91.

Peake, S. J., Venning, J. D., and Jackson, J. B. (1999) Biochim. Biophys. Acta 1411, 159–169.

Mejbaum-Katelennel, S., and Drubyszyczyk, W. J. (1969) Clin. Chem. 4, 513–523.

Venning, J. D., Rodrigues, D. J., Weston, C. J., Cotton, N. P. J., Quirk, P. G., Errington, N., Finet, S., White, S. A., and Jackson, J. B. (2001) J. Biol. Chem. 276, 30678–30685.

Bizouarn, T., Sanazov, L. A., Aubourg, S., and Jackson, J. B. (1996) Biochim. Biophys. Acta 1273, 4–12.

Cunningham, I. J., Williams, R., Palmer, T., Thomas, C. M., and Jackson, J. B. (1992) Biochim. Biophys. Acta 1100, 332–338.

Clayton, R. K. (1965) Biochim. Biophys. Acta 73, 312–323.

Venning, J. D., and Jackson, J. B. (1999) Biochem. J. 341, 329–337.

Mori, S., Abeygunawardana, C., Johnson, M. G., and Van Zijl, P. (1995) J. Magn. Res. Series B 108, 94–98.

Quirk, P. G., Jeeves, M., Cotton, N. P. J., Smith, K. J., and Jackson, J. B. (1999) FEBS Lett. 446, 127–131.

Leslie, A. G. W. (1992) J. Mol. Biol. 232, 531–543.

Cunningham, I. J., Williams, R., Palmer, T., Thomas, C. M., and Jackson, J. B. (1996) J. Mol. Struct. (Theochem.) 362, 407–416.

Cummins, P. L., and Gready, J. E. (1989) Biochim. Biophys. Acta 991, 91–100.

Buckley, P. A., Jackson, J. B., Schneider, T., White, S. A., Rice, D. W., and Baker, P. J. (2000) Structure 8, 809–815.

Prasad, G. S., Wahlberg, M., Sridhar, V., Sundaresan, V., Yamaguchi, M., Hafey, Y., and Stout, C. D. (1999) Nat. Struct. Biol. 6, 1126–1131.

Jackson, J. B., Peake, S. J., and White, S. A. (1999) FEBS Lett. 464, 1–8.

Weiss, S. A., Peake, S. J., Quinn, P. G., Cotton, N. P. J., and Jackson, J. B. (2000) Structure 8, 1–12.

Cotton, N. P. J., White, S. A., Peake, S. J., McSweeney, S., and Jackson, J. B. (2004) Structure 9, 165–170.

van Boel, G. L., Quirk, P., Cotton, N. P. J., and Jackson, J. B. (2003) Biochemistry 42, 1217–1226.

Jesch, M., Smith, R. K., Quinn, P. G., Cotton, N. P. J., and Jackson, J. B. (2000) Biochim. Biophys. Acta 1457, 211–218.

Hafey, Y., and Yamaguchi, M. (1996) FASEB J. 10, 444–452.

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REFERENCES

1. Rydstrom, J. and Hook, J. B. (1986) Biochem. J. 245, 1–10.

2. Sanazov, L. A., and Jackson, J. B. (1994) FEBS Lett. 344, 109–116.

3. Jackson, J. B., White, S. A., Quirk, P. G., and Venning, J. D. (2002) Biochemistry 41, 4173–4185.

4. Bizouarn, T., Rydstrom, O., Mueller, J., Axelson, M., Bergkvist, A., Johansson, C., Karlsson, G., and Rydstrom, J. (2000) Biochim. Biophys. Acta 1457, 211–218.

5. Hafey, Y., and Yamaguchi, M. (1996) FASEB J. 10, 444–452.