Senior, A. E. (1971) *J. Bioenerg. 2*, 141.
Solov'eva, A. A., & Yasaitis, A. A. (1974) *Biokhimiya 39*, 1212.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *Methods Enzymol. 10*, 448.
Sundler, R., Sarcione, S. L., Alberts, A. W., & Vagelos, P. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3350.
Tinberg, H. M., Packer, L., & Keith, A. D. (1972) *Biochim. Biophys. Acta 283*, 193.
Tinberg, H. M., Melnick, R. L., Maguire, J., & Packer, L. (1973) *Biochim. Biophys. Acta 311*, 230.
Tinberg, H. M., Melnick, R. L., Mcguire, J., & Packer L. (1974) *Biochim. Biophys. Acta 345*, 118.
Vignais, P. M., Nachbaur, J., Colbeau, A., & Vignais, P. V. (1971) *Adv. Exp. Med. Biol. 14*, 87.
Wehrle, J. P., Cintron, N. M., & Pedersen, P. L. (1978) *J. Biol. Chem. 253*, 8598.
Whiteley, N. M., & Berg, H. C. (1974) *J. Mol. Biol. 87*, 541.

Synthesis of a Spin-Labeled Analogue of Nicotinamide Adenine Dinucleotide Phosphate and Its Interaction with Dihydrofolate Reductase†

Lennie Cocco and Raymond L. Blakley*

ABSTRACT: A spin-labeled analogue of NADP has been prepared and characterized. Adenosine 2′,3′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy) has been prepared by coupling adenosine 2′,3′-phosphate 5′-phosphoromorpholidate with 4-phospho-2,2,6,6-tetramethylpiperidinyl-1-oxy and isolated by ion-exchange chromatography. The cyclic phosphate was cleaved by treatment with dilute acid to give a mixture of the 2′- and 3′-phosphate isomers. Incubation with a 3′-nucleotidase and subsequent ion-exchange chromatography gave the desired 2′-phosphate isomer, adenosine 2′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy). The spin-labeled analogue of NADP inhibits dihydrofolate reductase isoenzyme II from *Streptococcus faecium* var. *durans* Strain A. The inhibition is competitive with respect to NADPH with $K_{is} = 7.7 \pm 1.4$ μM. This is consistent with the dissociation constant for the inhibitor complex, $K_D = 5.1 \pm 0.8$ μM, as measured by the effect of binding on the EPR signal of the ligand. The electron paramagnetic resonance (EPR) measurements also show that the number of binding sites for the ligand on the reductase is 1.1 ± 0.1. The EPR signal shape at high ratios of the analogue to enzyme is unchanged from the signal in the absence of enzyme, but at low ratios of analogue to enzyme there is a marked broadening of the signal. Analysis of the enzyme-bound signal gave a correlation time of 5 ns, which is somewhat shorter than the rotational correlation of the enzyme [20 ns; Cocco, L., Blakley, R. L., Walker, T. E., London, R. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 4285–4290]. The analogue does not appear to inhibit glutamate dehydrogenase, isocitrate dehydrogenase, or glucose-6-phosphate dehydrogenase.

The structure of dihydrofolate reductase (EC 1.5.1.3) is the subject of intensive study because of its clinical relevance (Kisliuk & Brown, 1979; Matthews et al., 1978, 1979), the reductase being the target in cancer chemotherapy utilizing the drug methotrexate (Blakley, 1969). As part of continuing investigations of dihydrofolate reductase structure, we are using NMR spectroscopy of ^{13}C-labeled dihydrofolate reductase (Blakley et al., 1978; Cocco et al., 1978, 1979), which can yield information about motion of side chains, protein tumbling, conformational transitions, and ligand binding. In order to extract all the information from the NMR spectra, resonances need to be assigned to specific amino acid residues in the protein sequence.

A possible first step in making such assignments is the identification of resonances corresponding to residues near the active site. This would help to distinguish spectral changes reflecting conformational transitions from those due to direct interaction between the ligand and labeled residues. One tool for the identification of such resonances is the spin-labeled analogue of the ligands (Krugh, 1976; Morrisett, 1976). Resonances of ^{13}C nuclei near the bound spin-labeled ligand will be broadened, and in favorable cases the distance between the spin label and the ^{13}C nucleus can be calculated. In the present paper we report on the synthesis and characterization of a spin-labeled analogue of NADP to be used for this purpose. Some of these results have been presented in a preliminary form (Cocco & Blakley, 1978; Cocco et al., 1979). The corresponding spin-labeled analogue of NAD has been synthesized by Weiner (1969).

Experimental Procedure

Materials

4-Hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo-OH)[1] was obtained from the Aldrich Chemical Co. 2-

† From the Department of Biochemistry, College of Medicine, The University of Iowa, Iowa City, Iowa 52242. *Received December 20, 1978.* This work was supported in part by U.S. Public Health Service Research Grant CA 13840 from the National Cancer Institute of the National Institutes of Health.

[1] Abbreviations used: Tempo-OH, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy; PEI, poly(ethylenimine); DEAE, diethylaminoethyl; DCC, dicyclohexylcarbodiimide; cPADP-SL; adenosine 2′,3′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); PADP-SL, adenosine 2′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); Mes, 2-(*N*-morpholino)ethanesulfonic acid; ADP-SL, adenosine 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); TLC, thin-layer chromatography; BSA, bovine serum albumin; ADPR, adenosine 5′-diphosphoribose.

0006-2960/79/0418-2414$01.00/0

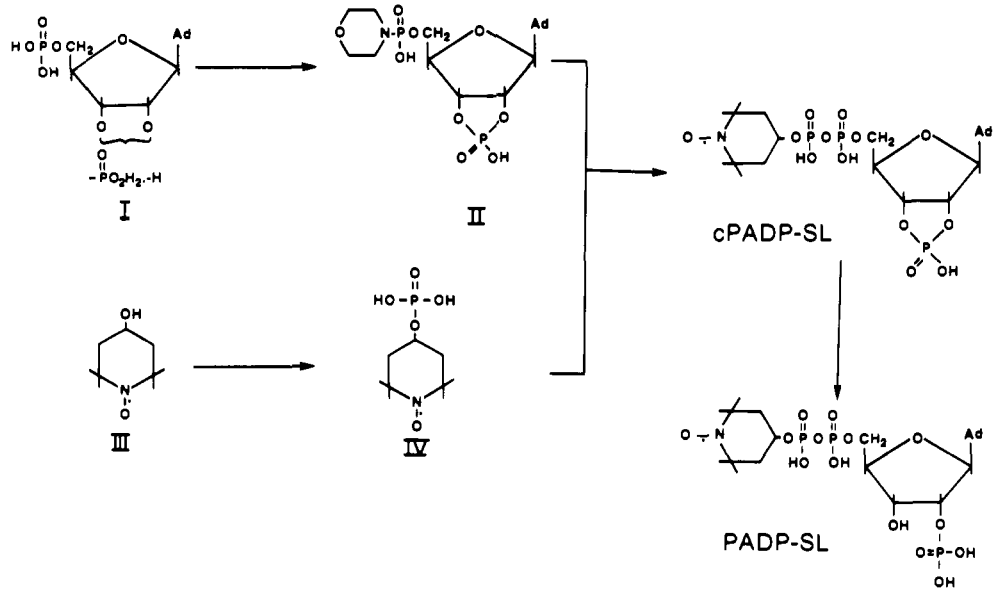

FIGURE 1: Outline of the method used for synthesis of PADP-SL.

Cyanoethyl phosphate, from the Sigma Chemical Co. (as the Ba salt), was converted to the free acid by the method of Tener (1961). *p*-(Methylamino)phenol (Elon) was from Kodak. Nuclease P_1 (EC 3.1.3.6), bovine liver glutamate dehydrogenase (EC 1.4.1.3, Type II, solution in 50% glycerol, 50 units/mg), pig heart isocitrate dehydrogenase (EC 1.1.1.42, Type IV, solution in 50% glycerol, 10 units/mg), and baker's yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Type VII, crystalline suspension, 300 units/mg) were obtained from Sigma. Thin-layer cellulose sheets were obtained from the Eastman Chemical Co. (Eastman Chromatogram Sheet 6065), and PEI-cellulose sheets were from Brinkman Instruments, Inc. (Polygram CEL 300 PEI/UV_{254}). Dowex-1, Dowex-50, and DEAE-cellulose were from Sigma.

Dihydrofolate reductase was purified as previously described (Blakley et al., 1978).

Methods

Analytical Procedures. TLC was carried out on cellulose sheets with saturated ammonium sulfate–ethanol (5:2) as the solvent (system 1) or on PEI-cellulose. For the latter 1.0 M LiCl was used as the solvent (system 2) after a preliminary development with water to remove salts and/or solvents from the sample. Spots were located under UV light.

Inorganic phosphate was determined by a modification of the method of Fiske and Subbarow by using 1% *p*-(methylamino)phenol containing 3% sodium bisulfite as the reducing agent (Jaenicke, 1974). Total phosphate was determined as inorganic phosphate after samples were ashed with $Mg(NO_3)_2$ (Ames & Dubin, 1960). The concentration of adenine derivatives was determined by using a molar absorbance of 15.4 $\times 10^3$ at 260 nm at pH 12. Ribose was determined by the method of Ashwell (1957).

Adenosine 2′(3′),5′-Diphosphate (I) (Figure 1). Adenosine (2.58 g, 10 mmol, dried overnight in vacuo at 100 °C) was dissolved in 100 mL of boiling anhydrous pyridine. The solution was allowed to cool, the solvent was removed in vacuo, 20 mL of a solution of the 2-cyanoethyl phosphate (20 mmol) in pyridine (Tener, 1961) was added, and the solvent was removed in vacuo. The residue was dissolved in 30 mL of anhydrous pyridine, and the solvent was again removed in vacuo. This was repeated twice more. A solution of 5.14 g (25 mmol) of dicyclohexylcarbodimide in 100 mL of anhydrous pyridine was added, and the flask was stoppered and shaken until the mixture became homogeneous. After 3 days at room temperature, 20 mL of water was added and the mixture allowed to sit for 1 h. The dicyclohexylurea was filtered off and washed with water. The resulting solution was evaporated to dryness in vacuo, and the residue was dissolved in 100 mL of 0.4 N LiOH and refluxed for 1 h. The cooled solution was filtered, diluted to about 1 L, and applied to a Dowex-1 ($HCOO^-$) (4 × 30 cm) column. The column was washed with about 4 L of water to elute unreacted adenosine. The monophosphorylated products were eluted with 1.25 M HCOOH (about 3 L) before elution of the 2′(3′),5′-ADP with 3.5 M HCOOH (about 3 L). The pooled fractions of the latter were evaporated to dryness in vacuo (water aspirator), the residue was redissolved in 200 mL of water, and evaporation to dryness was repeated. This procedure was repeated several times to remove residual formic acid. The remaining material which was homogeneous by TLC was used in this form for the next step. The yield was typically about 25%. TLC: system 1, R_f = 0.01; system 2, R_f = 0.08.

Adenosine 2′,3′-Phosphate 5′-Phosphoromorpholidate. The morpholidate (II) was prepared by the method of Moffatt & Khorana (1961a) with the modification that the DCC in *t*-BuOH was added dropwise to the reaction mixture over a period of 3–4 h (Moffatt & Khorana, 1961b). TLC: system 1, R_f = 0.60; system 2, R_f = 0.75. The TLC (system 2) showed two additional spots (trace contaminants compared to compound II) which corresponded to unreacted starting material and adenosine 2′,3′-phosphate 5′-phosphate. The UV spectrum showed a single absorbance maximum at 259 nm.

4-Phospho-2,2,6,6-tetramethylpiperidinyl-1-oxy (IV). Compound IV was prepared from Tempo-OH (III) by the method of Weiner (1969) and used as a 1 mM solution in pyridine. The material was homogeneous by TLC. TLC: III, system 2, R_f = 0.95; IV, system 1, R_f = 0.77; system 2, R_f = 0.78.

cPADP-SL. ADP morpholidate (II; 0.5 mmol) was dissolved in 20 mL of anhydrous pyridine, and the solvent was removed in vacuo. The residue was dried by three further evaporations with anhydrous pyridine. In another flask a solution of IV (2.0 mmol) was taken to dryness, and the residue was dried by three further evaporations with anhydrous pyridine. The residue of compound IV was taken up in 10 mL of anhydrous pyridine, the resulting solution was added to the morpholidate, and the solvent was removed in vacuo. After

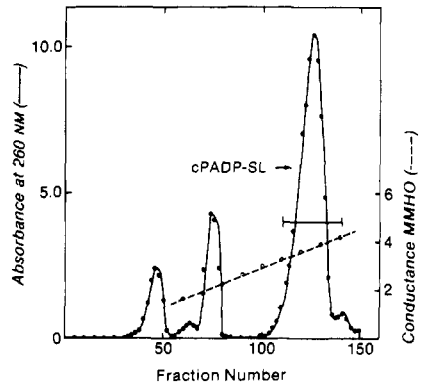

FIGURE 2: Purification of cPADP-SL by chromatography on DEAE-cellulose. Experimental details are in the text. Fractions combined are indicated by the bar.

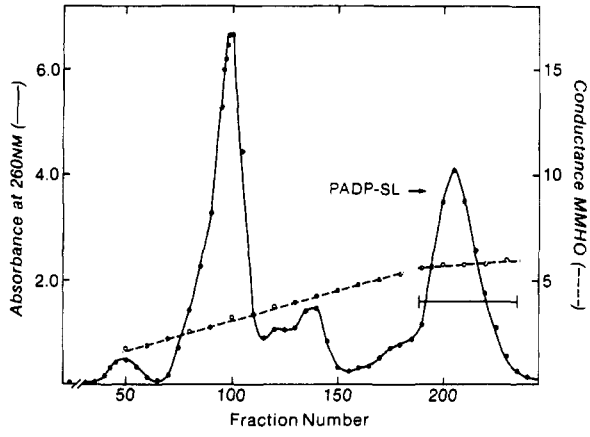

FIGURE 3: Purification of PADP-SL by chromatography on DEAE-cellulose. Fractions combined are indicated by the bar. Experimental details are in the text.

two more evaporations with anhydrous pyridine and readmission of dry air, 30 mL of anhydrous pyridine was added, the flask was stoppered, and the mixture was allowed to stand in the dark at 25 °C for 24 h. The solvent was removed in vacuo, and the residue was taken up in about 100 mL of water and applied directly to a DEAE-cellulose (bicarbonate) (3 × 45 cm) column. After the column had been washed with about 500 mL of water, a linear gradient was started (2 L of water in the stirred vessel and 2 L of 0.3 M triethylammonium bicarbonate, pH 7.3, in the reservoir), and 15-mL fractions were collected. A typical elution profile is shown in Figure 2. The major peak which contained the cyclic PADP-SL was dried in vacuo, and the buffer was removed by repeated evaporations with ethanol. The material which was homogeneous by TLC was used in this form for the next step. TLC: system II, R_f = 0.55. The UV spectrum showed a single maximum at 259 nm.

PADP-SL. The cPADP-SL was dissolved in 0.1 N HCl and allowed to sit for 1 h. The solution was neutralized with 1 N NaOH and made 0.05 M in Mes, pH 6.0, and about 5 mM in PADP-SL. Nuclease P_1 (0.5 mL of a 0.1 mg/mL solution) was added, and the mixture was incubated at 37 °C for 3–4 h. The solution was diluted 10-fold with cold water, applied to a DEAE-cellulose (bicarbonate) (3 × 30 cm) column, and washed with about 2 L of water. A linear gradient was started (2 L of water in the stirred vessel and 2 L of 0.4 M triethylammonium bicarbonate, pH 7.3, in the reservoir), and 15-mL fractions were collected. A typical elution profile is shown in Figure 3. Fractions of the second major peak were pooled. The first peak is ADP-SL while the second peak is PADP-SL. The PADP-SL sample was dried in vacuo, and

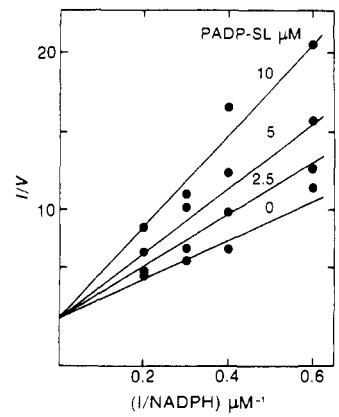

FIGURE 4: Double-reciprocal plot showing inhibition (competitive vs. NADPH) of dihydrofolate reductase with PADP-SL. Experimental details are in the text.

the buffer was removed as described above. The material was used without further purification. TLC: ADP-SL, system 2, R_f = 0.85; PADP-SL, system 1, R_f = 0.52; system 2, R_f = 0.46. The adenosine–ribose–phosphate–Tempo ratio was 1:1.2:3.5:0.7. A sample was converted to the tetrasodium salt by passage of a solution of PADP-SL over a Dowex-50 (Na^+) column. The solution was dried in vacuo, and the residue was dissolved in methanol. Several evaporations with the methanol were carried out to dry the material. Finally, a small volume of methanol was used to takeup the residue and solid PADP-SL precipitated from solution by addition of ~5 volumes of diethyl ether. The solid was filtered and air-dried on a Büchner funnel and then further dried over P_2O_5. Anal. Calcd for $C_{19}H_{28}N_6O_{14}P_3Na_4 \cdot 4CH_3OH$: C, 31.48; H, 5.05; N, 9.58. Found: C, 30.73; H, 4.88; N, 9.01.

Kinetics Measurements. Enzyme activities were measured spectrophotometrically with a Cary 14 recording spectrophotometer at 25 °C by using a 5-cm path length and the 0–0.1 absorbance scale and monitoring either the production or the loss of NADPH at 340 nm. For dihydrofolate reductase the assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 μM dihydrofolate, 1–5 μM NADPH, and 2–10 μM PADP-SL. The glutamate dehydrogenase assay mixture contained 10 mM Tris–acetate buffer (pH 8.0), 50 mM glutamate, 10 μM EDTA, 1–5 μM $NADP^+$, and 40 μM PADP-SL (Frieden, 1959). The isocitrate dehydrogenase assay mixture contained 30 mM triethanolamine hydrochloride buffer (pH 7.4), 4 mM DL-isocitrate, 20 mM $MnSO_4$, 1–5 μM $NADP^+$, and 45 μM PADP-SL (Coleman, 1968). The glucose-6-phosphate dehydrogenase assay mixture contained 100 mM Tris-HCl buffer (pH 8.0), 1 mM glucose 6-phosphate, 10 mM $MgSO_4$, 1–5 μM $NADP^+$, and 40 μM PADP-SL (Kuby & Noltmann, 1966).

Data were analyzed by Cleland's HYPER and COMP programs (Cleland, 1967).

EPR Measurements. EPR spectra were obtained at room temperature with either a Varian V-4502 or Varian E-104 spectrometer at 9.5 GHz. The spectrometer setting was time constant, 0.128 s; modulation amplitude, 0.63 G; microwave power, 20 mW; scan range, 100 G; scan time, 4 min; field, 3388 G; microwave frequency, 9.515 GHz; and temperature, 25 °C. All samples were in an aqueous flat cell. Samples used for the titrations contained 1 mg/mL BSA which did not affect the spectrum of the PADP-SL.

Results

Kinetic Behavior of the Analogue. PADP-SL was found to be a competitive inhibitor vs. NADPH for dihydrofolate

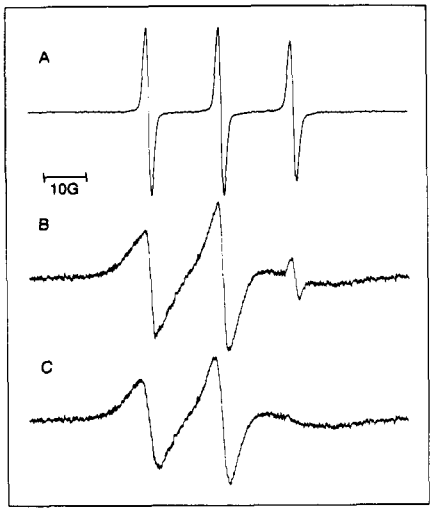

FIGURE 5: EPR spectra. (A) 50 μM PADP-SL; (B) 20 μM PADP-SL and 0.6 mM dihydrofolate reductase; (C) same as (B) with the small free signal removed from the entire spectrum by computer subtraction of an appropriately scaled spectrum of free PADP-SL. Samples are in 50 mM potassium phosphate buffer, pH 7.3. Spectrum B represents four scans collected with a time-averaging computer.

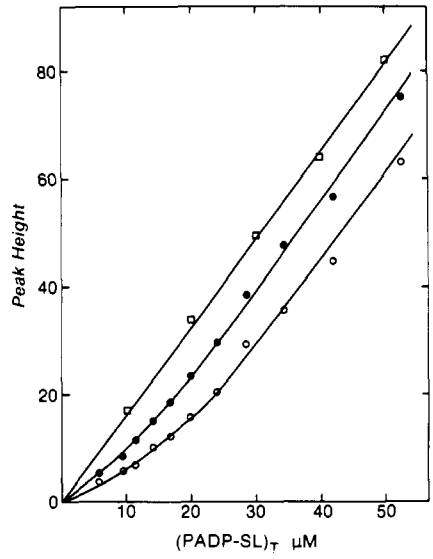

FIGURE 6: EPR titration of dihydrofolate reductase with PADP-SL. Curves are for PADP-SL with no enzyme (□), PADP-SL with 6.6 μM enzyme (●), and PADP-SL with 13.5 μM enzyme (○). All samples are in 50 mM potassium phosphate buffer, pH 7.3, containing 1 mg/mL BSA. (PADP-SL)$_T$ is the total concentration of PADP-SL in the sample.

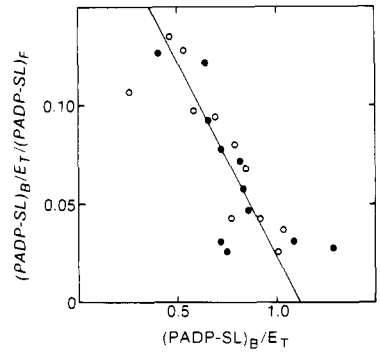

FIGURE 7: Scatchard plot of the EPR titration of dihydrofolate reductase with PADP-SL. Calculation is as described in text by using data taken from Figure 6. Enzyme concentrations were 6.6 μM (●) and 13.5 μM (○). (PADP-SL)$_B$, (PADP-SL)$_F$, and E_T are the concentrations of bound PADP-SL, free PADP-SL, and total enzyme, respectively.

reductase (Figure 4). The K_{is} at 25 °C was 7.7 ± 1.4 μM which is considerably lower than the K_{is} for PADPR (K_{is} = 28 μM; Blakley et al., 1978). With glutamate dehydrogenase, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase, inhibition was not observed at PADP-SL concentrations to 40 μM. On the assumption that in order to observe inhibition there must be a 20% decrease in the velocity, then $K_I > 4I$, where I is the inhibitor concentration used in the assay. Thus, the lower limit of K_I is 160 μM for the three dehydrogenases.

EPR Properties of the Analogue. PADP-SL has the typical three-line EPR spectrum expected for a paramagnetic nitroxide (Figure 5A). In the presence of dihydrofolate reductase there is a decrease in the intensity of the signal. At a high PADP-SL to enzyme ratio there is no change in line shape of the spectrum because the spectrum is dominated by the signal of the unbound PADP-SL. However, at low PADP-SL to enzyme ratio (i.e., <1) an altered shape is observed (Figure 5B,C).

By contrast, spectra of PADP-SL in the presence of glutamate dehydrogenase and isocitrate dehydrogenase show no line-shape indications of binding, even at low PADP-SL to enzyme ratios.

Titration of Dihydrofolate Reductase with PADP-SL. The dissociation constant of the complex formed by PADP-SL with dihydrofolate reductase was measured by titrating the enzyme with PADP-SL. It was assumed that the signal of the bound PADP-SL does not contribute significantly to the height of the upfield peak. Thus, the concentration of free ligand was estimated from the upfield peak height by comparison to a standard curve. The results of two titrations are shown in Figure 6.

The data from the titration were treated by the method of Scatchard (1949) to determine the K_D and number of binding sites (Figure 7). A linear least-squares analysis of these data (Cleland's LINE program) gave a K_D of 5.1 ± 0.8 μM and 1.1 ± 0.1 binding sites per molecule of reductase. These results are in good agreement with the kinetically derived value of K_{is}, and it may be seen that there is no evidence for a second binding site with $K_D < 25$ μM. This is in contrast to the report of two sites for the reductase of *Escherichia coli* with K_D = 0.02 and 0.4 μM, respectively (Williams et al., 1973).

Estimation of the Rotational Correlation Time (τ) for Bound PADP-SL. The spectrum of bound PADP-SL was obtained at an enzyme to PADP-SL ratio of about 30:1. Examination of the upfield portion of the spectrum (Figure 5b) showed about 3% contribution due to free PADP-SL. This contribution to the overall spectrum was removed by computer subtraction of an appropriately scaled signal for free PADP-SL. The resulting corrected spectrum of the bound PADP-SL (Figure 5c) can be used to estimate the rotation correlation time (τ) of the bound PADP-SL (Stone et al., 1965). The EPR spectrum consists of three lines assigned to ^{14}N nuclear spin quantum states M = 1, 0, and −1. The lines are of unequal width, and this inequality is a measure of the anisotropy of the hyperfine interaction and the g value. The heights of the lines of the derivative curve can be related to the rotational correlation time as follows (Stone et al., 1965)

$$\left(\frac{h_0}{h_{-1}}\right)^{1/2} - \left(\frac{h_0}{h_1}\right)^{1/2} = \frac{8\tau b \Delta\gamma H_0 T_{2,0}}{15} \quad (1)$$

$$\left(\frac{h_0}{h_{-1}}\right)^{1/2} + \left(\frac{h_0}{h_1}\right)^{1/2} = 2 + \left(\frac{2\tau b^2 T_{2,0}}{8}\right) \quad (2)$$

where h_M = heights of lines in the derivative curve, τ = the rotational correlation time, b = the measure of the anisotropy of the hyperfine interaction, $\Delta\gamma$ = the measure of the anisotropy of the g value, H_0 = magnetic field strength, and $T_{2,0}$ = spin–spin relaxation time estimate by using the peak-to-peak line width of the central line.

The values of b and $\Delta\gamma$ are calculated from constants for the free nitroxyl radical by using the equations (Stone et al., 1965)

$$b = \frac{4}{3}(A_{zz} - A_{xx}) \quad (3)$$

$$\Delta\gamma = -\frac{\beta}{h}g_{zz} - \frac{1}{2}(g_{xx} + g_{yy}) \quad (4)$$

where A_{zz} and A_{xx} = principal components of the hyperfine tensor, g_{xx}, g_{yy}, and g_{zz} = principal components of the g tensor, β = the Bohr magneton for the electron, and h = Planck's constant.

For the nitroxyl radical the appropriate constants are A_{zz} = 87 MHz, $A_{xx} = A_{yy}$ = 14 MHz, g_{zz} = 2.0027, g_{xx} = 2.0089, and g_{yy} = 2.0061 (Nordio, 1976); thus, $b = 3.06 \times 10^8$ rad and $\Delta\gamma = 4.22 \times 10^4$ s^{-1} G^{-1}. $T_{2,0}$ can be calculated from the peak-to-peak width ($\Delta\nu$, in gauss) of the central line by using the relation

$$(T_{2,0})^{-1} = \Delta\nu\pi\sqrt{3}(2.8 \times 10^6)\ \text{s}^{-1} \quad (5)$$

For PADP-SL $T_{2,0} = 18.1 \times 10^{-9}$ s; the peak heights were h_1 = 8.5, h_0 = 12.0, and h_{-1} = 1.3. Thus, $(h_0/h_{-1})^{1/2} = 3.04$ and $(h_0/h_1)^{1/2} = 1.19$, and eq 1 gave τ = 4.4 ns and eq 2 gave τ = 5.6 ns. The principle source of error in these estimates arises from the error introduced by spectrum noise in the estimate of h_{-1}. The maximum value of this error in h_{-1} was estimated to be ±25%, which would lead to a maximum error in τ of ±5%.

Discussion

Synthesis of PADP-SL. The structure of PADP-SL is the same as the NAD spin-labeled analogue synthesized by Weiner (1969), except for the 2′-phosphate group. Nevertheless, this difference introduced two synthetic problems: (1) the need to protect the 2′-phosphate by cyclization during the formation of the pyrophosphate bond between the piperidinyloxy moiety and the nucleotide and (2) the separation of the 2′ and 3′ isomers formed by the subsequent opening of the cyclic phosphate. The first problem was solved by using the same intermediate employed by Moffatt & Khorana (1961a) in the preparation of coenzyme A, adenosine 2′,3′-phosphate 5′-phosphoromorpholidate. In this compound the 5′-phosphate is activated and the 2′,3′-cyclic phosphate is protected. Allowing this material to react with 4-phospho-2,2,6,6-tetramethylpiperidinyl-1-oxy gives the spin-labeled analogue with the 2′-phosphate still protected. After chemical opening of the cyclic phosphate (with dilute acid), a mixture of the 2′ and 3′ isomers resulted, and attempts to separate these chromatographically were unsuccessful. However, use of a 3′-nucleotidase to remove the 3′-phosphate gave ADP-SL, the spin-labeled analogue of NAD, which is easily separated from PADP-SL. The synthetic scheme involving these procedures is conveniently carried out on a 1-mmol scale in about 2 weeks, giving about 150 μmol (~120 mg of the tetrasodium salt) of PADP-SL.

Suitability of PADP-SL for NMR Studies. A spin-labeled analogue for use in NMR studies of proteins must meet several criteria: (1) the dissociation constant should be low enough that a small excess of ligand gives nearly complete saturation of the protein; (2) the analogue should bind to the target protein so that its orientation on the protein surface is similar to that of the parent ligand; (3) any conformational changes induced by the parent ligand should also be induced by the analogue; and (4) the nitroxyl portion of the bound analogue should also be located near the active site of the protein. Presumably, these criteria were also considered in designing a spin-labeled analogue of NAD (Weiner, 1969).

The choice of the particular spin-labeled analogue reported on here was based on earlier observations of the behavior of PADPR with dihydrofolate reductase. PADPR is a competitive inhibitor vs. NADPH and binds tightly to the catalytic site (K_{is} = 28 μM; Blakley et al., 1978). Similarly, PADP-SL was found to inhibit dihydrofolate reductase competitively vs. NADPH, and its dissociation constant was found to be low ($K_D \approx 7$ μM) when determined both kinetically and by EPR titration. At a 1:1 ratio of PADP-SL to enzyme, at least 92% of the enzyme is saturated, so that the relatively small amounts of unbound PADP-SL should cause no significant nonspecific broadening of resonances in the NMR experiments. In addition, the EPR titration results show that there is only one binding site (at PADP-SL to enzyme ratios of 1:1); thus, there will be no complications due to PADP-SL at a second, lower affinity site.

The kinetics of inhibition (competitive vs. NADPH) suggest that PADP-SL binds to dihydrofolate reductase in a manner similar to NADPH. Furthermore, binary complexes of [*guanidino*-^{13}C]arginine-labeled dihydrofolate reductase with NADPH and with PADP-SL give very similar ^{13}C NMR spectra (if broadening of some resonances is neglected),[2] which is taken as evidence that the two ligands are binding in a similar orientation on the protein surface and also inducing similar conformational transitions.

Interaction of PADP-SL with Other Dehydrogenases. Since dihydrofolate reductase is inhibited by both PADP-SL and PADPR, this enzyme presumably interacts similarly with the nicotinamide ribose and the piperidinyloxy group. The same similarity of behavior was observed with ADPR and ADP-SL toward liver alcohol dehydrogenase (Weiner, 1969). However, the piperidinyloxy group apparently does not interact in the same manner as the nicotinamide ribose in the case of other NADP-linked dehydrogenases. Thus, glutamate dehydrogenase, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase, all of which are inhibited by PADPR (Ben-Hayyim et al., 1967), showed no detectable inhibition by PADP-SL. The binding sites of the larger dehydrogenases therefore seem to have more steric constraints than does the site of dihydrofolate reductase or are limited in the types of interactions allowed. Comparison of space-filling models of the ribose group and the piperidinyloxy group shows that they occupy nearly the same volume, so that any difference in binding must derive from the differences in hydrophilicity. The three-dimensional structure of *Lactobacillus casei* dihydrofolate reductase shows that the nicotinamide ribose binding site is relatively open and can accommodate either hydrophobic or hydrophilic groups (Matthews et al., 1979). In agreement with this conclusion, the correlation time for PADP-SL bound to the reductase (see below) is consistent with a loosely attached piperidinyloxy group.

When the nicotinamide binding sites of lactate dehydrogenase and dihydrofolate reductase are compared, however, there are no evident resemblances. The former has binding surfaces for the nicotinamide on some β sheets and

[2] Unpublished experiments.

α helixes which comprise a mononucleotide fold, whereas many interactions with the nicotinamide in the latter involve residues which are not part of β-sheet or α-helical structure (Matthews et al., 1979). Furthermore, of the three larger dehydrogenases studied, only glucose-6-phosphate dehydrogenase has an ordered mechanism[3] (Gould & Goheer, 1976) as does dihydrofolate reductase (Blakley et al., 1971), whereas glutamate dehydrogenase (Engel & Dalziel, 1970) and isocitrate dehydrogenase (Uhr et al., 1974; Northrup & Cleland, 1974) have random mechanisms. This also suggests that the larger dehydrogenases do not interact with NADPH in the same manner as dihydrofolate reductase.

Correlation Time of Bound PADP-SL. Estimation of the correlation time from the EPR spectrum of the bound PADP-SL gives a value of about 5 ns, which is significantly smaller than the value of 20 ns determined for dihydrofolate reductase by NMR methods (Cocco et al., 1978). This difference probably reflects some mobility of the piperidinyloxy moiety in the active site generating internal motion with a faster rate than protein tumbling. Differences in the value of τ calculated from eq 1 and 2 have been attributed to either anisotropic motion or solvent dependences of the hyperfine and g tensors (Stone et al., 1965). In the case examined by Campbell et al. (1972), even smaller relative differences in τ calculated by the two methods were considered to be indicative of anisotropic motion. Inspection of eq 1 and 2 reveals that the τ values will be equal only when h_1 and h_0 are equal. This is the case with the free PADP-SL but is clearly not so with enzyme-bound spin label.

Acknowledgments

We thank Drs. G. R. Buettner, G. Kapke, L. W. Oberly, and J. Baron for their assistance with the electron paramagnetic resonance measurements.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Ashwell, G. (1957) *Methods Enzymol. 3*, 73–105.

Ben-Hayyim, G., Hockman, A., & Avron, M. (1967) *J. Biol. Chem. 242*, 2837–2839.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, pp 157–176, North-Holland Publishing Co., Amsterdam.

Blakley, R. L., Schrock, M., Sommer, K., & Nixon, P. F. (1971) *Ann. N.Y. Acad. Sci. 186*, 119–130.

Blakley, R. L., Cocco, L., London, R. E., Walker, T. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 2284–2293.

Campbell, I. D., Dwek, R. A., Price, N. C., & Radda, G. K. (1972) *Eur. J. Biochem. 30*, 339–347.

[3] Glucose-6-phosphate dehydrogenase from most sources has a random mechanism, and the results for the bakers' yeast enzyme must be considered tentative (Levy, 1979).

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Cocco, L., & Blakley, R. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1811.

Cocco, L., Blakley, R. L., Walker, T. E., London, R. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 4285–4290.

Cocco, L., Blakley, R. L., London, R. E., Groff, J. P., Walker, T. E., & Matwiyoff, N. A. (1979) in *Chemistry and Biology of Pteridines*, pp 383–388, Elsevier/North-Holland, New York.

Coleman, R. F. (1968) *J. Biol. Chem. 243*, 2454–2464.

Engel, P. C., & Dalziel, K. (1970) *Biochem. J. 118*, 409–419.

Frieden, C. (1959) *J. Biol. Chem. 234*, 809–814.

Gould, B. J., & Goheer, M. A. (1976) *Biochem. J. 157*, 389–393.

Jaenicke, L. (1974) *Anal. Biochem. 61*, 623–627.

Kisliuk, R. L., & Brown, G. M., Eds. (1979) *Chemistry and Biology of Pteridines*, pp 353–355, 357–359, 361–366, 419–424, 437–440, 465–470, 471–476, 483–488, Elsevier/North-Holland, New York.

Krugh, T. R. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 339–372, Academic Press, New York.

Kuby, S. A., & Noltmann, E. A. (1966) *Methods Enzymol. 9*, 116–125.

Levy, H. R. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 97–192.

Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N. H., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946–6954.

Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N. H., & Kraut, J. (1979) *J. Biol. Chem.* (in press).

Moffatt, J. G., & Khorana, H. G. (1961a) *J. Am. Chem. Soc. 83*, 663–675.

Moffatt, J. G., & Khorana, H. G. (1961b) *J. Am. Chem. Soc. 83*, 649–658.

Morrisett, J. D. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 274–338, Academic Press, New York.

Nordio, P. L. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) p 31, Academic Press, New York.

Northrup, D. B., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2928–2931.

Scatchard, T. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010–1017.

Tener, G. M. (1961) *J. Am. Chem. Soc. 83*, 159–168.

Uhr, M. L., Thompson, V. W., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2920–2927.

Weiner, H. (1969) *Biochemistry 8*, 526–533.

Williams, M. N., Greenfield, N. J., & Hoogsteen, K. (1973) *J. Biol. Chem. 248*, 6380–6386.