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Dihydrofolate Reductase: Multiple Conformations and Alternative Modes of Substrate Binding†

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Received July 14, 1988; Revised Manuscript Received October 28, 1988

ABSTRACT: The complex of *Lactobacillus casei* dihydrofolate reductase with the substrate folate and the coenzyme $NADP^+$ has been shown to exist in solution as a mixture of three slowly interconverting conformations whose proportions are pH-dependent [Birdsall, B., Gronenborn, A. M., Hyde, E. I., Clore, G. M., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1982) *Biochemistry 21*, 5831]. The assignment of the resonances of all the aromatic protons of the ligand molecules in all three conformational states of the complex has now been completed by using a variety of NMR methods, particularly two-dimensional exchange experiments. The resonances of the nicotinamide protons of the coenzyme and the pteridine 7-proton of the folate have different chemical shifts in the three conformations, in some cases differing by more than 1 ppm. Comparison of the COSY spectra of the complex at low pH (conformation I) and high pH (conformations IIa and IIb) with that of the enzyme–methotrexate–$NADP^+$ complex shows only slight differences in the conformation of the protein. The pattern of chemical shift changes in the ligand and the protein indicates that the structural differences are localized within the active site of the enzyme. Nuclear Overhauser effects (NOEs) are observed between the nicotinamide 5- and 6-protons and the methyl resonance of Thr 45 at both low and high pH, indicating that there is no major movement of the nicotinamide ring. By contrast, NOEs are observed between the pteridine 7-proton and the methyl protons of Leu 19 and Leu 27 in conformations I and IIa but *not* in conformation IIb. A model is proposed that can qualitatively account for the observed NOEs and ligand ^1H chemical shifts. In this model, the folate pteridine ring in conformations I and IIa is bound in a way similar to that observed for the pteridine ring of methotrexate in the crystal structure of the enzyme–methotrexate–NADPH complex. In conformation IIb, however, it has a quite different orientation in the binding site, related to that in states I and IIa by a rotation of about 180° about an axis approximately along the C2–NH_2 bond. This latter orientation would account for the observed stereochemistry of reduction of folate by the enzyme. It thus appears that folate is able to bind to the enzyme both in a *productive* and in a quite different *nonproductive* mode, while the inhibitor methotrexate binds only in the nonproductive mode.

Dihydrofolate reductase, which is responsible for maintaining the cellular pools of tetrahydrofolate derivatives, is an NADPH-linked dehydrogenase that catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate. It is inhibited by the important "anti-folate" drugs such as trimethoprim and methotrexate. In recent years, a substantial body of structural information on complexes of the enzyme with inhibitors has become available from X-ray crystallography and NMR spectroscopy [for review, see Roberts (1983), Beddell (1984), Freisheim and Matthews (1984), Blakley (1985), and Feeney (1986)]. Much less information is available concerning substrate binding and the catalytic mechanism. However, studies of the stereochemistry of the reaction have established that in the catalytically functional complex the substrate must bind in an orientation substantially different from that seen for methotrexate in the crystal (Hitchings & Roth, 1980; Charlton et al., 1979, 1985). Thus, in spite of the close structural similarity between folate and methotrexate, the only important difference being the replacement of the 4-oxo substituent of folate by an amino group in methotrexate, there appear to be marked differences in their modes of interaction with the enzyme.

Kinetic and NMR experiments have provided evidence that under a variety of conditions dihydrofolate reductase exists in solution as a mixture of two or more conformational states that interconvert only slowly. This appears to be the case for the enzyme alone (Pattishall et al., 1976; Dunn et al., 1978; London et al., 1979; Cayley et al., 1981) and for the enzyme–trimethoprim–$NADP^+$ (Gronenborn et al., 1981a,b) and

† We are grateful to the Medical Research Council for the award of a Training Fellowship to S.J.B.T. and to the SERC and the Wellcome Trust for support of the work at Leicester.
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0006-2960/89/0428-2297$01.50/0

enzyme–folinic acid–coenzyme complexes (Birdsall et al., 1981a). In particular, we have shown that complexes of the *Lactobacillus casei* enzyme with folate, in the presence or absence of $NADP^+$, exist in solution in two or three conformational states whose proportions are pH-dependent (Birdsall et al., 1981b, 1982, 1987), while there is no evidence that more than one conformation of the enzyme–methotrexate or enzyme–methotrexate–coenzyme complexes is substantially populated (Hammond et al., 1986).

We now report further NMR experiments on the dihydrofolate reductase–folate–$NADP^+$ complex which allow us to begin to define the nature of the differences between the three conformations of this complex and which throw light on the relationships between folate and methotrexate binding. A preliminary account of part of this work has appeared (Roberts, 1987).

Materials and Methods

L. casei dihydrofolate reductase was isolated either from *L. casei* MTX/R or from *Escherichia coli* containing a plasmid bearing the *L. casei* dihydrofolate reductase gene under the control of the λ p_L promoter (Andrews et al., 1989) and was purified as described by Dann et al. (1976). Its concentration was determined by assaying its catalytic activity and by fluorometric titration with methotrexate (Dann et al., 1976), and it was shown to be free of coenzyme by UV and NMR spectroscopy. Folate, $NADP^+$, and NADPH were obtained from Sigma Chemical Co.

NMR Spectroscopy. ^1H NMR spectra were obtained at 400 and 500 MHz by using Bruker AM400 and AM500 spectrometers. Samples consisted of 1–3 mM enzyme, with 0–13 molar equiv of folate and/or coenzyme, in ^2H_2O containing 50 mM potassium phosphate, either 100 or 500 mM KCl, 1 mM EDTA, and 1 mM dioxane, adjusted to pH* 5.0–7.7 by careful addition of ^2HCl or NaO^2H. (The notation pH* indicates measured pH values in ^2H_2O uncorrected for the ^2H isotope effect on the glass electrode.) Magnitude COSY and phase-sensitive NOESY and exchange spectra were obtained as described previously (Hammond et al., 1986). In general, 1024 data points were recorded in t_2 for each of 256–512 t_1 values, averaging 128–256 transients for each free induction decay. The data were zero-filled as required to give 1024 or 2048 points in each dimension and processed with a phase-shifted sine bell function for the NOESY spectra or a sine-squared bell function for the COSY spectra; two-dimensional Fourier transformation produced spectra with a digital resolution of 6–12 Hz/point.

The multisite irradiation transfer of saturation experiments were performed by saturating two resonances in the spectrum alternately for 50 ms each and repeating this 10 times to give a total irradiation time of 1 s before the observe pulse. A difference spectrum was formed by subtracting an "experimental" spectrum, in which free and bound signals were saturated, and a "control" spectrum, in which the free signal and a control site were saturated. The order of irradiation of the free and bound signals and of the free and control sites was matched.

The stereochemistry of reduction of folate at pH* 5.2 was determined by using $NADP^2H$ as described previously (Charlton et al., 1979, 1985).

Results

Chemical Shifts of Ligand Resonances. We first demonstrated the existence of three conformations of the dihydrofolate reductase–folate–$NADP^+$ complex by ^{13}C NMR studies with [*carboxamide*-^{13}C]$NADP^+$ (Birdsall et al., 1981b). At low pH (<5.5) a single conformation, denoted conformation I, was observed, while at high pH (>7.5) two other conformations, denoted IIa and IIb, were found to coexist in a ratio of 1:1.67. At intermediate pH values, all three forms are present. We subsequently used ^1H transfer of saturation experiments at 270 MHz and selectively ^2H-labeled coenzyme to identify and assign many of the ^1H resonances of the bound $NADP^+$ and the 7-proton resonance of the bound folate in this complex (Birdsall et al., (1982). However, for only two of these protons, N2 and N4, was it possible to identify unambiguously resonances from all three conformational states. (Protons of the nicotinamide and adenine moieties of the coenzyme are denoted N and A, respectively.)

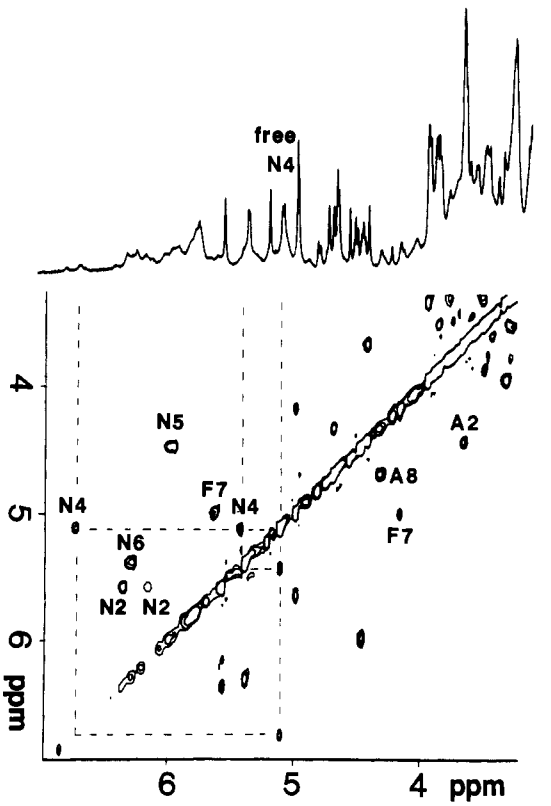

FIGURE 1: Aromatic region of a two-dimensional ^1H NMR spectrum obtained from a sample containing dihydrofolate reductase and a 2-fold molar excess of both $NADP^+$ and folate at pH* 6.5, using the NOESY pulse sequence with a mixing time of 100 ms. The corresponding region of the one-dimensional spectrum is shown above. Under the conditions of this experiment, the cross-peaks shown arise from exchange of the ligands between the free state and conformations IIa and IIb of the complex. The ligand proton from which each cross-peak arises is indicated; N2–N6 denote the protons of the nicotinamide ring and A2, A8 the protons of the adenine ring of $NADP^+$, while F7 denotes the pteridine 7-proton of folate.

We have now been able to complete the assignment of the aromatic and anomeric ^1H resonances of the bound ligands by using two-dimensional (2D) exchange spectroscopy and one-dimensional transfer of saturation experiments at 500 MHz. Figure 1 shows the aromatic region of a 2D spectrum obtained with the NOESY pulse sequence by using a mixing time of 100 ms from a sample containing dihydrofolate reductase and a 2-fold molar excess of both $NADP^+$ and folate at pH* 6.5. Under these conditions, the majority of the intense cross-peaks arise from chemical exchange of the ligand molecules between the free and the bound states. Exchange cross-peaks can be distinguished unambiguously from nuclear Overhauser effect cross-peaks by the fact that they are present only when the sample contains excess free ligand. As can be seen from Figure 1, two distinct exchange cross-peaks, from

Table I: Chemical Shifts of Ligand Nuclei in the Three Conformational States of the Dihydrofolate Reductase–Folate–$NADP^+$ Complex and in the Dihydrofolate Reductase–Methotrexate (MTX)–$NADP^+$ Complex

	chemical shift[a] (ppm)			
	enzyme–folate–$NADP^+$			enzyme–MTX–$NADP^+$[b]
nucleus	I	IIa	IIb	
nicotinamide (^1H, ^{13}C)				
N2	6.32	6.30	6.12	6.34
N4	6.81	6.68	5.38	6.16
N5	5.74	5.92[c]	5.96[c]	5.34
N6	6.41	6.24	6.24	6.31
$^{13}CONH_2$	96.32	95.88	94.26	95.96[d]
pyrophosphate (^{31}P)	−14.4	−14.4	−14.4	−14.9
	−16.3	−16.3	−16.3	−16.5
adenine (^1H, ^{31}P)				
A2	3.63	3.60	3.60	3.64
A8	4.30	4.26	4.26	4.31
$2'PO_4$	2.7	2.7	2.7	2.7
pteridine (^1H, ^{15}N)				
H7	4.50	4.12	5.58	4.50
$^{15}N5$	2.5	9.9	10.7	
2′,6′H	2.25	1.84	1.84	
3′,5′H	3.37	3.26	3.26	

[a] Nicotinamide and adenine protons of the coenzyme are denoted N2–N6 and A2, A8, respectively. ^1H and ^{13}C chemical shifts are given relative to dioxane, ^{15}N shifts relative to the resonance of free folate, pH* 6.3, and ^{31}P shifts relative to inorganic phosphate, pH* 8.0. Downfield shifts positive. ^{13}C and ^{31}P data from Birdsall et al. (1982), ^{15}N data from Birdsall et al. (1987). ^1H chemical shifts have been redetermined in the present work and are more precise than those obtained earlier from transfer of saturation experiments at 270 MHz (Birdsall et al., 1982). [b] Data from Hyde et al. (1980a,b). [c] Individual assignment to conformations IIa and IIb is uncertain. [d] Data (recalculated relative to dioxane) from Way et al. (1975).

conformations IIa and IIb, are seen for the nicotinamide 2-, 4-, and 5-protons (denoted N2, N4, and N5, respectively) and for the 7-proton of folate (denoted F7); the two N5 cross-peaks are partly overlapping. Only single resonances were identified in this experiment for the N6 and N1′ protons and for the adenosine protons A2, A8, and A1′ of the bound coenzyme. Although one must be careful in interpreting the *absence* of cross-peaks, the simplest explanation is that the chemical shifts of these protons are the same in conformations IIa and IIb of the complex, since in the free ligands the T_1 values of the protons for which only single cross-peaks are observed are similar to or longer than those of protons for which two distinct cross-peaks are seen.

While conformations I, IIa, and IIb are all significantly populated at this pH (Birdsall et al., 1982), exchange cross-peaks are seen in this experiment only between free ligand and states IIa and IIb because the ligands dissociate too slowly from state I of the complex. In order to locate the ligand resonances from state I, we have studied the complex at pH* 5.3, by transfer of saturation experiments at 270 and 500 MHz and by 2D exchange experiments at 500 MHz. We have also located some of the resonances from state I by selective deuteration experiments at 270 MHz (Birdsall et al., 1982). The results of these experiments are summarized in Table I, which lists the measured ^1H chemical shifts for all three states of the complex, together with the ^{13}C, ^{15}N, and ^{31}P chemical shifts reported earlier (Birdsall et al., 1981b, 1982, 1987) and the corresponding values for the enzyme–methotrexate–$NADP^+$ complex.

When more than one form of the complex is present, the intensities of the cross-peaks in 2D exchange experiments do not give a reliable indication of the amounts of the different conformers present. In fact, the rate of exchange between the free and bound states can be a much more important determinant of the magnitude of the observed exchange cross-peak or transfer of saturation effect than the relative populations of the conformational forms (Clore et al., 1981). Thus one cannot use the cross-peak intensities to assign ligand signals to specific conformational states of the complex. The assignments of the chemical shifts of a given proton of a bound ligand to conformations I, IIa, or IIb indicated in Table I are based on the pH dependence of the intensity of resonances which can be observed directly; an example is shown in Figure 2 for the N4 resonances from states I and IIa. Although many more of the bound ligand signals could be resolved and observed directly at 500 MHz than at 270 MHz, in most cases it was still only possible to resolve signals from two of the three conformations. However, since all the available evidence is consistent with the conclusion from the ^{13}C spectra (Birdsall et al., 1981b) that the population ratio IIb/IIa has a pH-independent value of 1.67, direct observation of two of the three signals is sufficient to identify the conformations from which they arise.

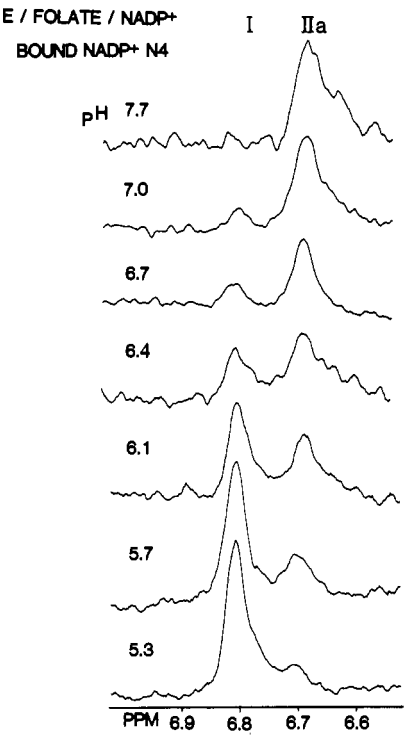

FIGURE 2: Resonances of the nicotinamide 4-proton in conformations I and IIa of the dihydrofolate reductase–folate–$NADP^+$ complex as a function of pH*. The resonance of this proton in conformation IIb is substantially further upfield, at 5.38 ppm, and is not shown in these spectra.

The 2D NOESY/exchange experiment also allows one to observe transferred nuclear Overhauser enhancement (NOE) effects (Albrand et al., 1979). The observation of transferred NOE cross-peaks between N1′ and N2 (but not between N1′ and N6) for state IIa demonstrates that the bound coenzyme has the anti conformation about the nicotinamide glycosidic bond in this state of the enzyme–folate–$NADP^+$ complex as it does in the enzyme–methotrexate–$NADP^+$ complex (Albrand et al., 1979; Feeney et al., 1983). However, it was not possible to say with certainty whether these NOEs were present in conformations I and IIb of the enzyme–folate–$NADP^+$ complex.

Chemical Shifts of Assigned Protein Resonances. We have identified a limited number of amino acid residues whose resonances show differences in chemical shift between two or more of the conformations of the enzyme–folate–$NADP^+$

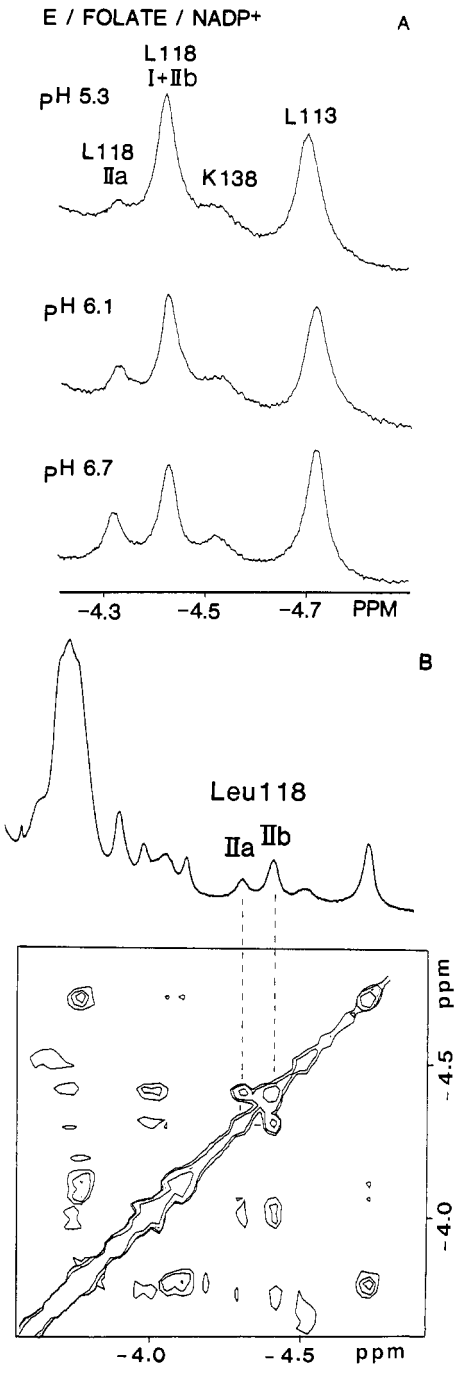

FIGURE 3: (A) ^{1}H resonances of one methyl group of leucine 113, one methyl group of leucine 118, and one β-proton of lysine 138 of the dihydrofolate reductase–folate–NADP$^+$ complex as a function of pH*. (B) Corresponding region of a two-dimensional ^{1}H NMR spectrum of the complex obtained with the NOESY pulse sequence at pH* 6.5; the cross-peaks arising from the exchange of the methyl protons of Leu 118 between conformations IIa and IIb are indicated.

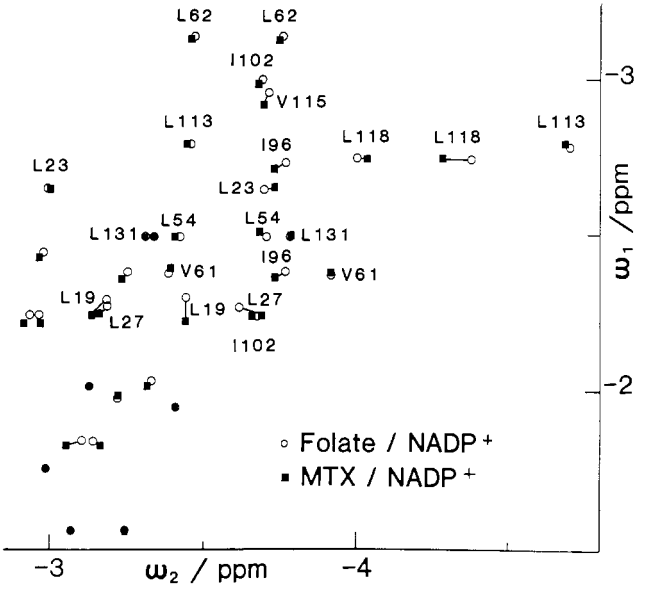

FIGURE 4: Schematic comparison of the methyl region of the two-dimensional ^{1}H COSY spectra of the dihydrofolate reductase–folate–NADP$^+$ and dihydrofolate reductase–methotrexate–NADP$^+$ complexes, both at pH* 5.

complex. In an earlier paper (Birdsall et al., 1982) we showed that two histidine $C_{\epsilon 1}$–H resonances, now firmly assigned to histidines 18 and 22 (Hammond et al., 1986), each appeared as *two* signals at pH* values above 6.5. For histidine 18, cross-peaks linking the two $C_{\epsilon 1}$–H signals are observed in a 2D exchange experiment (not shown), confirming that this proton has different chemical shifts in conformations IIa and IIb.

Figure 3A shows the highest field resonances in the ^{1}H spectrum of the complex at three pH* values. Taking the methyl resonance of Leu 113 as an intensity reference, it can be seen that the methyl resonance of Leu 118 has two components whose relative intensities are pH-dependent. The 2D exchange experiment (Figure 3B) demonstrates that these two signals do indeed come from the same methyl group in two distinct environments. Since the higher field signal is essentially the only one observed at pH* 5.3, it must represent the Leu 118 methyl group in conformation I. However, this higher field signal does not disappear as the pH is increased but rather reaches a constant ratio to the lower field signal. This can readily be explained if the lower field component of the Leu 118 resonance represents conformation IIa and the higher field component *both* conformations IIb and I.

Figure 4 shows a schematic comparison of the high-field region of the 2D COSY spectra of the enzyme–folate–NADP$^+$ complex at pH* 5.0 (i.e., in conformation I) and the enzyme–methotrexate–NADP$^+$ complex at the same pH* (the spectrum of the enzyme–methotrexate–NADP$^+$ complex is pH-independent over the range pH* 5–7). This comparison shows that, as already suggested by the ^{1}H chemical shifts of the bound coenzyme in Table I, these two complexes are structurally very similar, though not identical. Since the chemical shifts in these two complexes are so similar, we have transferred the assignments made for the enzyme–methotrexate–NADP$^+$ complex (Hammond et al., 1986) to the corresponding folate complex on the basis of similarity in both chemical shift and the pattern of COSY connectivities. Differences in chemical shift of more than 0.05 ppm are seen only for Leu 4, Leu 19, Leu 23, Leu 27, Ile 96, and Leu 118, and these are summarized in Table II. We have also obtained COSY spectra of the enzyme–folate–NADP$^+$ complex at high pH, where it exists as a mixture of conformations IIa and IIb. It is apparent from these spectra that the chemical shifts of the vast majority of protein resonances are very similar in all three conformations of the complex, although a clear distinction between cross-peaks arising from conformations IIa and IIb could only rarely be made with certainty (e.g., Leu 118; see Table II).

Ligand–Protein NOE Experiments. In 2D NOESY spectra of the enzyme–folate–NADP$^+$ complex, clear cross-peaks are observed between the N5 and N6 resonances of the bound coenzyme and a signal assigned (B. Birdsall, unpublished work) to the methyl protons of Thr 45 (not shown). These

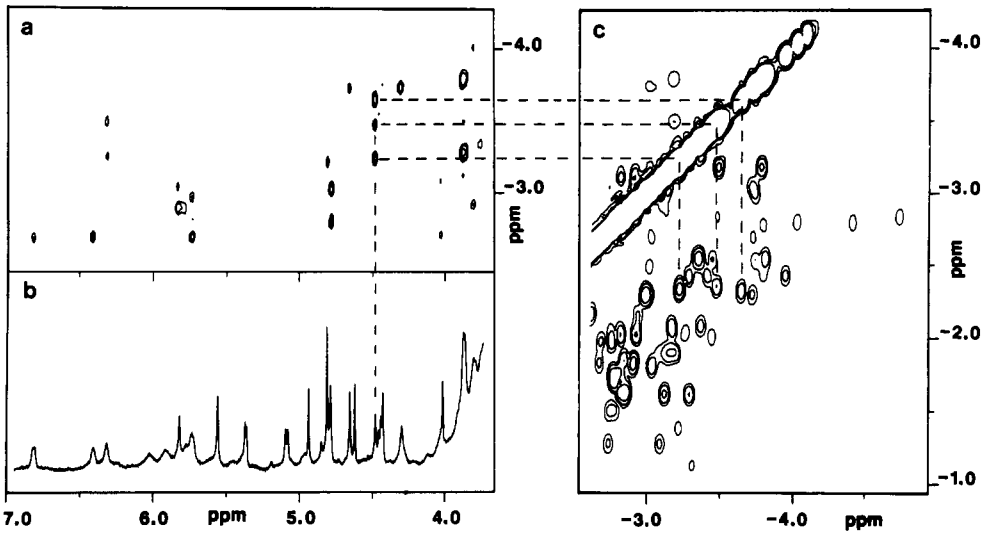

FIGURE 5: Nuclear Overhauser effects involving the folate pteridine 7-proton and methyl groups of the protein in conformation I of the dihydrofolate reductase–folate–NADP$^+$ complex (pH* 5). Spectrum b is the low-field part of the aromatic region of the one-dimensional ^{1}H spectrum of the complex, with the resonance of the 7-proton of bound folate indicated. Part a shows the region of the two-dimensional NOESY spectrum of the complex containing cross-peaks between low-field aromatic protons and methyl protons. Part c shows the high-field part of the two-dimensional ^{1}H COSY spectrum of the complex. The dashed lines connect the resonance of the the folate 7-proton in (b) with its three NOESY cross-peaks in (a) and the corresponding COSY cross-peaks in (c).

Table II: Chemical Shifts of Some Assigned ^{1}H Resonances of the Protein in Conformation I of the Dihydrofolate Reductase–Folate–NADP$^+$ Complex and in the Dihydrofolate Reductase–Methotrexate–NADP$^+$ Complex[a]

		chemical shift[b] (ppm)			
		enzyme–folate–NADP$^+$			enzyme–MTX–NADP$^+$[d]
residue	proton	I[c]	IIa[c]	IIb[c]	
Leu 4	$C_\delta H_3$	−3.20			−3.14
	$C_\delta H_3$	−2.53			−2.55
	$C_\gamma H_3$	−1.37			−1.29
Leu 19	$C_\delta H_3$	−3.46			−3.46
	$C_\delta H_3$	−3.21			−3.16
	$C_\gamma H$	−2.34			−2.25
Leu 23	$C_\delta H_3$	−3.71			−3.75
	$C_\delta H_3$	−3.02			−3.02
	$C_\gamma H$	−2.67			−2.72
Leu 27	$C_\delta H_3$	−3.63			−3.71
	$C_\delta H_3$	−3.21			−3.16
	$C_\gamma H$	−2.34			−2.30
Ile 96	$C_\delta H_3$	−3.78			−3.75
	$C_\gamma H$	−2.76			−2.85
	$C_\gamma H$	−2.42			−2.41
Leu 118	$C_\delta H_3$	−4.39	−4.43	−4.34	−4.30
	$C_\delta H_3$	−4.01	−3.99	−4.05	−4.05
	$C_\gamma H$	−2.78	−2.80	−2.80	−2.77

[a] Data are given only for residues one or more of whose assigned resonances differ in chemical shift by more than 0.05 ppm between the enzyme–methotrexate–NADP$^+$ complex and conformation I of the enzyme–folate–NADP$^+$ complex; in the case of Leu 118, data are given for all three conformations of the latter complex. [b] Relative to dioxane. [c] I, IIa, and IIb refer to conformations I, IIa, and IIb, respectively. [d] From Hammond et al. (1986).

NOE cross-peaks are observed in spectra obtained both at low pH, where conformation I predominates, and at high pH, where conformation IIb has the highest population, indicating that the position of the nicotinamide ring in its binding site is very similar in these two conformational states.

In the enzyme–methotrexate–NADP$^+$ complex, NOEs are observed between the pteridine 7-proton of the inhibitor and the methyl protons of Leu 19 and Leu 27 [Hammond et al. (1986) and unpublished work]. The corresponding experiment for the enzyme–folate–NADP$^+$ complex, at pH* 5 where the complex will be very largely in conformation I, is summarized in Figure 5. Figure 5b shows the aromatic region of the spectrum of this complex, and Figure 5a the part of the 2D NOESY spectrum containing cross-peaks between aromatic and methyl protons. The resonance frequency of the 7-proton resonance of bound folate (identified by transfer of saturation) is indicated by a dashed line in both parts a and b. It can be seen that there are clear NOESY cross-peaks between the resonance of the 7-proton in conformation I and the resonances of three methyl groups. Figure 5c shows the methyl region of a 2D COSY spectrum of this complex, with the cross-peaks of these methyl resonances indicated. Comparison of the chemical shifts and COSY connectivity patterns of these resonances with those in the enzyme–methotrexate–NADP$^+$ complex shows that they arise from, in decreasing order of chemical shift, the higher field of the two methyl resonances of Leu 27, the higher field of the two methyl resonances of Leu 19, and the lower field methyl resonances of leucines 19 and/or 27. The lower field methyl ($C_\delta H_3$) and the $C_\gamma H_2$ resonances of leucines 19 and 27 overlap in the spectrum of conformation I of the enzyme–folate–NADP$^+$ complex (see Figure 4). We conclude that in conformation I of the complex the 7-proton of folate is close to (within about 4 Å of) one or both methyl groups of Leu 19 and Leu 27.

The corresponding data for pH* 6.5, where all three conformations are present, are shown in Figure 6. Part b of the figure shows the 2D exchange experiment which locates the resonances of the folate 7-proton in each of the three conformations. (Note that, under the conditions of this experiment, a weak exchange cross-peak is seen for conformation I as well as those for conformations IIa and IIb). Figure 6a shows that part of the NOESY spectrum which contains cross-peaks between aromatic and methyl protons. Following up the lines corresponding to the resonance frequencies of the folate 7-proton identified in the lower panel, it can be seen that, above −3 ppm, there are two NOESY cross-peaks arising from the 7-proton in each of conformations I and IIa. (In this experiment only two NOESY cross-peaks are seen in this region for conformation I, compared to three in Figure 5, due to the lower concentration of this conformation at higher pH.) The similarities in the chemical shifts and the COSY connectivity patterns (shown in Figure 6c) suggest that the same methyl groups are close to the pteridine 7-proton in both conformation I and conformation IIa of the folate complex

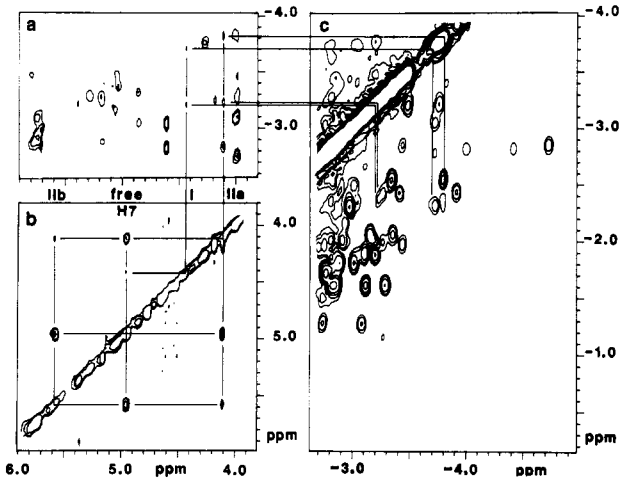

FIGURE 6: Nuclear Overhauser effects involving the folate pteridine 7-proton and methyl groups of the protein in the three conformations of the dihydrofolate reductase–folate–NADP$^+$ complex (pH* 6.5). Part b shows the aromatic region of the two-dimensional exchange spectrum obtained from a sample containing the complex and a 2-fold excess of free folate. The cross-peaks linking the 7-proton resonance of free folate at 4.95 ppm to the resonances of the same proton in the three conformations of the complex are connected by lines. Part a shows the region of the two-dimensional NOESY spectrum of the complex containing cross-peaks between aromatic and methyl proton resonances. Cross-peaks involving the 7-proton resonance in conformations I and IIa are indicated; there are no cross-peaks in this region of the spectrum involving the 7-proton resonance in conformation IIb. Part c shows the high-field region of the ^{1}H COSY spectrum of the complex. The lines connect the resonances of the 7-proton in conformations I and IIa of the complex (in part b) with their NOESY cross-peaks in part a and with the corresponding COSY cross-peaks in part c.

as well as in the methotrexate complex. By contrast, there is no evidence in the upper left-hand panel for an NOE between the folate 7-proton in conformation IIb and any methyl protons. As indicated above in connection with Figure 1, care is required when interpreting the *absence* of cross-peaks; it is likely, however, that the methyl and folate protons have similar T_1 values in the three conformations, and certainly their resonance line widths, where these can be measured, are essentially identical. Furthermore, since the population of conformation IIb is greater than that of IIa, the absence of cross-peaks in the former cannot be due to a concentration difference. Much the simplest interpretation of the absence of H7–methyl NOE cross-peaks in conformation IIb is that this reflects a greater distance (more than about 4 Å) between these protons in this conformation than in the other two. This must result from a structural (conformational) difference in the immediate vicinity of the pteridine ring of the bound folate molecule.

Exchange Mechanism. Since conformations IIa and IIb differ significantly in the environment of the pteridine ring of the bound folate, it is of interest to establish whether or not they can interconvert directly. In the 2D exchange experiment shown in panel b of Figure 6, as well as the cross-peaks between the 7-proton resonance of free folate and those of the three bound states, a weak cross-peak can be seen between the 7-proton resonances in conformations IIa and IIb; however, this leaves open the possibility that interconversion can take place only through free folate. To distinguish between direct and indirect interconversion between states IIa and IIb, we have carried out a series of one-dimensional transfer of saturation experiments at pH* 7.25 (where form I of the complex is not detectable). In an attempt to detect direct transfer of saturation between the two bound states, we compared the decrease in intensity of the folate 7-proton signal from state IIb on irradiating *both* the corresponding state IIa signal and the free 7-proton signal with that observed when *only* the free signal was irradiated. With a sample containing a concentration of folate equal to that of the enzyme, in which the concentration of *free* folate was thus very low, a clear decrease in intensity of the state IIb signal due to transfer of saturation from IIa was observed. When the folate concentration was increased to 2 molar equiv, the effect was much smaller, while at a concentration of 13 molar equiv no transfer of saturation between states IIa and IIb could be detected. This marked effect of the concentration of free folate on the apparent rate of exchange between states IIa and IIb demonstrates that the kinetically preferred pathway of exchange between these two states is through free folate.

Stereochemistry of Reduction. We have earlier described studies of the stereochemistry of reduction of folate to tetrahydrofolate at pH* 6.8, using NADP^{2}H as substrate and showing that the 7-*pro-S* proton in the tetrahydrofolate product was replaced by deuterium (Charlton et al., 1979, 1985). In view of the evidence for a pH-dependent difference in the orientation of the folate molecule in the active site, we have now used the same method to examine the stereochemistry of folate reduction at pH* 5. We find that the stereochemistry of the reaction is the same as that reported earlier for neutral pH conditions, namely, that the 4-*pro-R* hydrogen of NADPH is transferred to the *si* face at C7 of the pteridine ring of folate.

Discussion

The chemical shifts reported here and in earlier papers (Birdsall et al., 1981b, 1982, 1987), together with the NOE experiments, allow us to propose a model for the differences between the three conformational states of the dihydrofolate reductase–folate–NADP$^+$ complex. Considering first the coenzyme chemical shifts summarized in Table I, it is immediately apparent that the only nuclei whose environments differ between the conformations are those at the nicotinamide end of the molecule. Fewer signals from the bound folate molecule have been identified, but there seems to be a similar trend within its binding site, by far the largest differences being seen for the pteridine H7 and ^{15}N5. These data clearly focus our attention on the surroundings of the nicotinamide and pteridine rings—that is, on the center of the active site—as the likely source of the conformational difference between the three conformational states of the complex.

The ligand–protein NOE experiments confirm unambiguously the indications from the chemical shifts that, while the environment of the pteridine 7-proton is similar in states I and IIa, it is quite different in state IIb. In the former states the 7-proton is within 4 Å of two (conformation IIa) or three (conformation I) methyl groups, from leucines 19 and 27, while in state IIb it is much further from any methyl proton. In the enzyme–methotrexate–NADPH complex (Bolin et al., 1982), the pteridine ring of methotrexate binds to the enzyme in a predominantly hydrophobic "slot". Given the evidence, from the close similarity between the two-dimensional ^{1}H spectra of the three conformations of the enzyme–folate–NADP$^+$ complex and that of the enzyme–methotrexate–NADP$^+$ complex, that there is no major change in protein conformation, the shape of this binding pocket places considerable constraints on the possible modes of binding of folate. Thus, the observation that the pteridine 7-protons of both methotrexate and folate are close to the same two amino acid residues implies that the orientation of the two pteridine rings in the binding pocket must be the same. This indicates that in states I and IIa folate has a similar mode of binding to that observed for methotrexate in the crystal structure of the enzyme–metho-

trexate–NADPH complex (Bolin et al., 1982).

In state IIb the folate 7-proton appears to be more than about 4 Å from the methyl groups of Leu 19 and Leu 27, as indicated by the absence of NOEs between them. This observation cannot be accommodated within the steric constraints of the binding site if the folate pteridine ring binds in the same general orientation as that of methotrexate and only the limited lateral reorientation possible within the "slot" is allowed. It can, however, be accounted for within these constraints if the pteridine ring binds "upside down"—that is, rotated by 180° about an axis approximately coincident with the C2–NH_2 bond [see Bolin et al. (1982)]. This orientation can be made sterically acceptable by an appropriate adjustment of the C6–C9–N10–C1′ segment of the molecule, and this adjustment also allows the benzoylglutamate moiety to bind in the same way, as required by the observation that the resonance of His 28, which forms an ion pair with the γ-carboxylate, has the same chemical shift in all three conformations of the complex. This change in orientation of the pteridine ring has the effect of keeping the "pyrimidine" part of the ring in the same place in the binding site (although in a different orientation), but significantly altering the position of the "pyrazine" part.

Some further support for this proposed reorientation comes from the fact that it can provide a qualitative explanation for the pattern of ligand ^1H chemical shift differences between conformations IIa and IIb. The nicotinamide ring of the coenzyme is close to the pteridine ring, and the ring current anisotropy of the latter will contribute to the chemical shifts of the nicotinamide protons. Taking the position of the pteridine and nicotinamide rings in conformation IIa as those observed in the crystal structure of the enzyme–NADPH–methotrexate complex (Bolin et al., 1982), and the position of the pteridine ring in conformation IIb as described above, the difference in the ring current contribution to the chemical shift of the nicotinamide protons between the two conformations can be estimated. The calculated values (positive numbers for downfield shifts in IIa relative to IIb) are N2, 0.14; N4, 2.59; and N5, 0.22 ppm, compared to the experimental values of N2, 0.18; N4, 1.30; and N5, (±)0.04 ppm. Although the magnitude of the large effect on N4 is not well reproduced (other contributions to the shift difference have been neglected, and there is uncertainty as to the *precise* orientation of the pteridine ring in the two states), it is notable that the very selective effect on N4 as opposed to N2 and N5 is accounted for by this simple calculation. Other models, such as that in which the ring is rotated by 180° about the C2–C6 axis, fail to reproduce this observation. The chemical shift of the pteridine 7-proton has a large contribution from the anisotropy of the benzoyl ring of methotrexate or folate [see Hammond et al. (1987)], and the change in the mutual orientation of these two rings involved in the suggested mode of binding of folate in conformation IIb would certainly lead to a substantial downfield shift in the resonance of this proton, as observed experimentally. It is more difficult to account for the chemical shift of the $^{15}N5$ resonance of folate. This is similar in conformations IIa and IIb, but very different in conformation I; it is closest to the value in free folate, in which it is presumably hydrogen-bonded to water, in conformation I. However, in the crystal structure of the enzyme–methotrexate complex (Bolin et al., 1982) N5 does not form any hydrogen bonds, either to groups on the protein or to bound water molecules; in the absence of a proper understanding of the various contributions to the ^{15}N chemical shift, it is not possible to draw any definite conclusion.

By contrast to this marked difference in pteridine ring orientation, the observation of NOEs between the nicotinamide protons and Thr 45 in both conformations I and IIb indicates that the position of the nicotinamide in its binding site is similar in both cases, while the ^{31}P NMR spectrum (Birdsall et al., 1982) shows that this is also true of the conformation of the pyrophosphate "backbone" of the bound coenzyme and of the position of the 2′-phosphate group. In particular, it is clear that the differences between the three conformational states of the enzyme–folate–$NADP^+$ complex do not involve major differences in coenzyme binding of the kind that we have identified for the two conformational states of the enzyme–trimethoprim–$NADP^+$ complex (Gronenborn et al., 1981b; Birdsall et al., 1984).

The proposed 180° reorientation of the pteridine ring in conformation IIb relative to that in the crystal structure of the enzyme–methotrexate–NADPH complex is exactly that required to account for the observed stereochemistry of reduction of folate (Hitchings & Roth, 1980; Charlton et al., 1979, 1985). The mode of binding of folate that we propose for conformation IIb of the enzyme–folate–$NADP^+$ complex thus corresponds to the *productive* mode of binding in the catalytically functional enzyme–folate–NADPH complex. The alternative orientation seen in conformations I and IIa apparently represents a nonproductive mode of binding of the substrate, since we have now shown that the stereochemistry of folate reduction at low pH, where conformation I predominates, is the same as that determined previously at neutral pH. The striking difference between the binding of methotrexate seen in the crystal and the mode of binding of folate implied by the stereochemistry of reduction thus does not mean that the inhibitor binds in a unique way which is unavailable to the substrate. Rather, methotrexate binds essentially exclusively in only one of the two modes of binding available to the substrate, that corresponding to "nonproductive" substrate binding. The preference of methotrexate for this orientation must arise, at least in part, from the formation of an ion pair between the protonated pteridine ring and the carboxylate of Asp 26 (Bolin et al., 1982; Cocco et al., 1981) which is possible only in this orientation.

The nature of the ionizable group responsible for the pH dependence of the relative populations of the three conformational states of the enzyme–folate–$NADP^+$ complex, which is predicted to have $pK \leq 5$ in states IIa and IIb and $pK \geq 7$ in state I (Birdsall et al., 1982), is as yet unknown. However, histidine residues can be ruled out (Birdsall et al., 1982), and Asp 26 is the only ionizable amino acid side chain within 10 Å of the nicotinamide carboxamide carbon in the crystal structure of the enzyme–methotrexate–NADPH complex (Bolin et al., 1982), making it a plausible candidate. We have recently shown (Birdsall et al., 1988) that the substitution of Asp 26 by Glu does not affect the relative populations of the three conformations; a definitive test of the involvement of Asp 26 must await the results of studies of the Asn 26 enzyme currently in progress (J. Andrews, M. A. Jimenez, J. R. P. Arnold, J. A. Thomas, B. Birdsall, J. Feeney, and G. C. K. Roberts, unpublished work). A possible alternative candidate for the ionizable group giving rise to the pH dependence of the conformational equilibria is N3–H of folate itself; in the favored keto tautomer of folate, this nitrogen deprotonates with a pK of 8.38 (Poe, 1977), and it might be expected to have a quite different pK value in the two proposed orientations of the pteridine ring. Studies designed to test this possibility by using isotopically labeled folate are in progress.

The implications of the existence of two modes of binding of the substrate for the catalytic mechanism of the enzyme are not clear, since we do not know whether these two alternative modes of binding exist in the catalytically functional enzyme–folate–NADPH or enzyme–dihydrofolate–NADPH complexes. However, our recent observation of two distinct resonances for the ^{15}N5 and ^{1}H7 of folate in the binary enzyme–folate complex (Birdsall et al., 1987), with chemical shift differences similar to those between conformations I and IIb of the ternary enzyme–folate–NADP$^+$ complex, shows that the existence of these different conformational states of the ternary complex is not dependent on the presence of NADP$^+$. If this pH-dependent conformational equilibrium does exist in the catalytically functional complexes, the presence of a significant amount of nonproductive substrate binding would be expected to lower the apparent k_{cat} and to affect its pH dependence. In fact, the pH dependence of the fractional population of conformation IIb of the enzyme–folate–NADP$^+$ complex, with the pteridine ring bound in the productive orientation, is opposite to the pH dependence of k_{cat} for folate reduction by NADPH, which increases with decreasing pH (J. Andrews, C. Fierke, and S. Benkovic, unpublished work). However, even if the same conformational equilibria do occur in the catalytically functional enzyme–folate–NADPH complex, a simple correlation would not necessarily be expected since proton transfers are involved in the catalytic mechanism. More detailed kinetic studies of folate reduction, and NMR experiments on complexes more closely resembling the catalytic complexes, will be required to settle this point.

It is widely assumed that the reduction of the N5–C6 bond of dihydrofolate is promoted by transfer of a proton to N5 (Huennekens & Scrimgeour, 1964; Gready, 1985) from Asp 26, probably via a water molecule (Hitchings & Roth, 1980; Bolin et al., 1982; Freisheim & Matthews, 1984). Although there is as yet no clear-cut evidence for this proton transfer, substitution of this aspartate residue by asparagine does lead to a substantial decrease in catalytic activity (Villafranca et al., 1983; Howell et al., 1986; J. Andrews et al., unpublished work). Protonation of N5 of folate would be less favorable (Temple & Montgomery, 1985) and would not obviously promote the reduction of the C7–N8 bond, which is much the slower of the two consecutive enzyme-catalyzed reductions required to convert folate to tetrahydrofolate. In the stereochemically required mode of binding of folate, there appears to be no direct path for proton transfer from enzyme side chains or bound water molecules to N8, to promote reduction of the C7–N8 bond [see also Freisheim and Matthews (1984)]. It is possible to envisage a mechanism in which the "methotrexate-like" mode of binding of the substrate does play a role in the mechanism, by permitting protonation of N8 by Asp 26. This would require interconversion between the two orientations of folate after protonation at N8 but before hydride ion transfer to C7. The observation that, at least in the enzyme–folate–NADP$^+$ complex, the interconversion between the two orientations of the pteridine ring takes place through free folate makes such a mechanism improbable. The alternative of proton transfer from Asp 26 to O4 [see, e.g., Freisheim and Matthews (1984) and Gready (1985)] is perhaps more likely but is not yet supported by any experimental evidence. It remains possible that the proton transferred to N8 in the course of folate reduction comes directly from the solvent and that Asp 26 is not a proton donor for this reduction.

Acknowledgments

We are grateful to Gill Ostler and John McCormick for invaluable technical assistance. NMR spectra were obtained by using the AM400 and AM500 spectrometers of the MRC Biomedical NMR Centre.

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A New Electron Spin Resonance Assay for Membrane Asymmetry and Entrapped Volume of Unilamellar Lipid Vesicles Based on Photoreduced Flavin Adenine Dinucleotide†

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Received July 14, 1988; Revised Manuscript Received October 11, 1988

ABSTRACT: A new ESR assay has been developed for the characterization of unilamellar lipid vesicles. It is based on the reduction by photogenerated $FADH_2$ of amphiphilic spin-labels having the spin in the polar group. $FADH_2$ is generated in situ under anaerobic conditions from its oxidized form (FAD) by photoreduction in the presence of excess EDTA as the reducing agent. Photoreduction is induced by exposing the FAD/EDTA mixture to white light of a commercial slide projector. $FADH_2$ as an impermeable agent reduces spin-label molecules located on the outer layer of the bilayer that are readily accessible in a first fast reaction; spin-label located on the inner layer of the bilayer is reduced in a second slow reaction. The ESR assay is suitable for the routine characterization of unilamellar membrane vesicles: it allows the determination of the vesicle size, the entrapped volume, the bilayer asymmetry, the bilayer integrity, and the vesicle stability. The ESR assay developed is of general applicability: it can be used with charged and uncharged bilayers which may be labeled with either neutral or charged spin-labels. An assessment of the new ESR assay is given in comparison to the existing ascorbate method which uses sodium ascorbate as the reducing agent. Various other potential reducing agents for spin-labels have been tested and found unsuitable for the ESR assays discussed here.

A number of physicochemical methods have been employed and also new ones have been developed in the past decades that are suitable for the characterization of lipid bilayers and membrane vesicles. Among these methods ESR spin-labeling has been used succesfully to determine the fraction of spin-labeled lipid molecules located on the external surface of lipid bilayers and, related to it, the asymmetry in the transverse lipid distribution. Other applications of ESR techniques comprise the determination of the transverse bilayer motion or "flip-flop" of spin-labeled molecules (Kornberg & McConnell, 1971), the thermodynamic stability and integrity of bilayers of multilamellar and unilamellar vesicles (Strauss & Hauser, 1986), and the volume fraction entrapped in lipid vesicles (Marsh et al., 1976; Marsh & Watts, 1981). This entrapped volume is directly related to the encapsulation properties of lipid and membrane vesicles. Its routine determination is important when vesicles are used as carrier systems, e.g., in drug delivery.

All the spin-labeling methods mentioned above have in common that the spin-label or at least part of it is reduced to the corresponding hydroxylamine or other nonparamagnetic compounds under the action of the reducing agent sodium ascorbate (Smith et al., 1976; Marsh & Watts, 1981):

$$2{>}N\text{—}\dot{O} + Na^+ \text{ ascorbate} + H^+ \rightarrow 2{>}N\text{—}OH + Na^+ + \text{dehydroascorbate} \quad (1)$$

The use of sodium ascorbate as the reducing agent in ESR

† This work was supported by the Swiss National Science Foundation (Grant 3.511-0.85).
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0006-2960/89/0428-2305$01.50/0