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The inter-ligand Overhauser effect: A powerful new NMR approach for mapping structural relationships of macromolecular ligands

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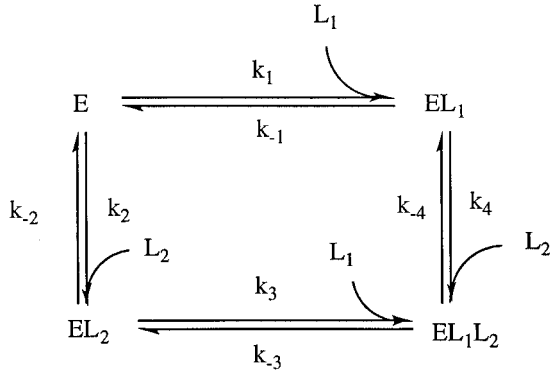
Abstract

NMR experiments that transfer conformational information from the bound to the uncomplexed state via exchange have been utilized for many years. It is demonstrated here that inter-ligand NOEs ('ILOEs'), which exist in ternary complexes with enzymes or other macromolecular receptors, can be transferred via exchange to pairs of uncomplexed ligands. This approach is illustrated by studies of glycolate + NAD^+ in the presence of porcine heart lactate dehydrogenase, and by glucose-6-phosphate + NADPH in the presence of *L. mesenteroides* glucose-6-phosphate dehydrogenase. This strategy opens up a general methodology for exploring the active sites of enzymes and for the development of artificial ligands which can function as inhibitors, or more generally as modifiers of protein function.

Enzymes catalyze bimolecular reactions by bringing pairs of reactants together and creating an environment which facilitates the reaction. When pairs of substrates or substrate–cofactor combinations are complexed in the active site of an enzyme, some of the nuclei on the two ligands may be sufficiently close to interact magnetically, leading to a perturbation of the NMR relaxation parameters and to an internuclear Overhauser effect. One may then consider the extent to which such an inter-ligand Overhauser effect in the ternary complex could be 'transferred' to the pair of uncomplexed ligands due to chemical exchange (Figure 1). Thus, we here consider the possibility that a transferred inter-ligand Overhauser effect or ILOE could be observed as a general phenomenon for a non-productive ternary complex involving two or more ligands binding to a macromolecular receptor. In contrast with the more traditional transferred nuclear Overhauser effect, which provides conformational information (Albrand et al., 1979; Gronenborn and Clore, 1990; Campbell and Sykes, 1991; Lippens et al., 1992; London et al., 1992; Nirmala et al., 1992; Ni, 1994; Moseley et al., 1995), such an experiment provides structural information on the relative orientation of ligands bound to the active site of an enzyme or other macromolecular receptor, even in the absence of direct information about the structure of the receptor. Further, this concept is readily generalized to the binding of ligands which are not directly related to substrates or cofactors, and hence to the development of enzyme inhibitors.

The quantitative prediction of ILOE build-up curves is based on a kinetic model in which two distinct ligands can simultaneously bind to a receptor, as illustrated below:



where E, EL_1, EL_2, and EL_1L_2 represent the uncomplexed receptor, the binary complexes with L_1 and

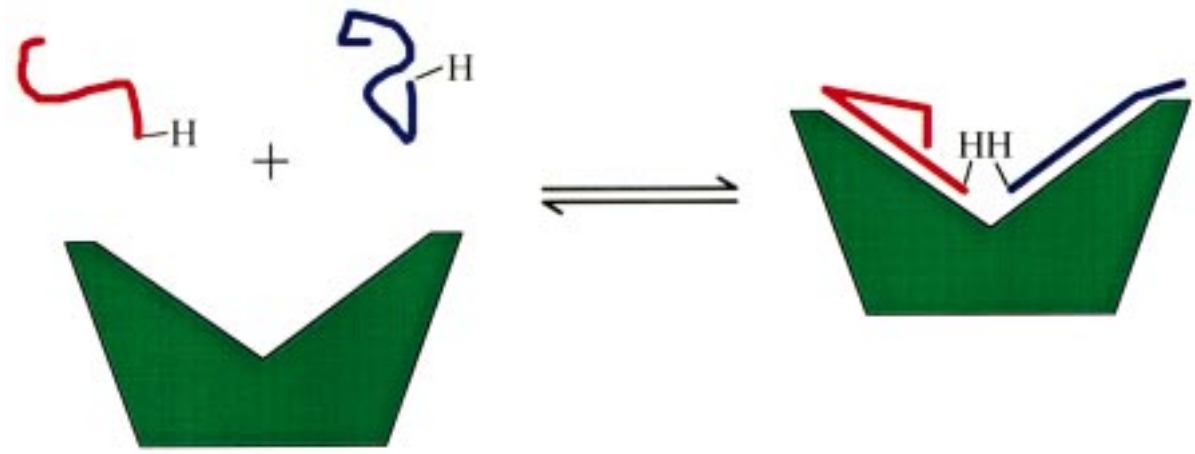


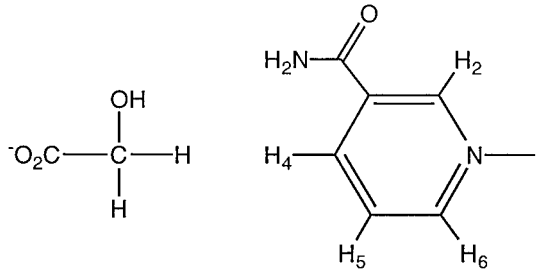
Figure 1. The formation of a ternary complex between a receptor and a pair of ligands. As indicated, some of the protons on the ligands may be sufficiently close to interact magnetically, leading to nuclear Overhauser effects, which can be transferred to the pair of free ligands due to dissociation of the complex. This forms the basis for the observation of inter-ligand Overhauser effects (ILOEs).

L_2 and the ternary complex. Such a kinetic model involves eight kinetic rate constants, one of which is constrained by mass balance. Analogous calculations based on a model requiring ordered ligand binding have also been performed. Theoretical simulations for various ligand geometries indicate that ILOE build-up curves are qualitatively similar to transferred NOE curves. For sufficiently rapid exchange rate constants, typical ILOE build-up curves display the same initial $1/r_{ij}^6$ dependence as the usual NOE curves, and can develop cross peak intensities which are an appreciable fraction of the transferred NOE peaks calculated for the same r_{ij} values. However, initial slopes derived from ILOE peaks can be compared to intra-ligand transferred NOE data only if the fractional occupancies of the receptor for the ternary complex and for the ligand used as a reference are very similar. Detailed simulations of the effect are given elsewhere (London, 1999).

In order to experimentally validate the existence of significant ILOE interactions, we have initially considered a series of dehydrogenases. This selection was based on several factors, particularly: (1) formation of ternary pyridine nucleotide–substrate complexes in which the pyridine ring is positioned close to the substrate in order to facilitate hydride ion transfer; (2) existence of a significant body of literature on transferred NOE studies of pyridine nucleotide-binding enzymes; (3) commercial availability. In setting up such experiments, it is necessary to use a stable system with no or negligible turnover. There are several ways of achieving this, including: (1) use of a pair of reduced or oxidized substrates, rather than the normal catalytic complex which will include one oxidized and one reduced substrate; (2) replacement of substrate and/or cofactor with an inhibitor; (3) analysis of a catalytic complex of an inactive enzyme produced via site-directed mutagenesis.

This approach is illustrated in Figure 2 for the ternary complex formed by NAD^+, and glycolate (a non-substrate analog of lactate) in the presence of porcine heart lactate dehydrogenase, a tetrameric enzyme of 140 kDa (Bernhardt et al., 1981; Grau et al., 1981). This enzyme is capable of reducing glyoxylate to glycolate in the presence of NADH; however, the enzyme-NAD^+-glycolate complex, if it forms, appears to be non-productive (Lluis and Bozal, 1977). This result was verified by NMR studies which demonstrated negligible disappearance of the glycolate proton resonance in the presence of NAD^+ and lactate dehydrogenase over a period of 12 days at 15 °C. The upper diagonal portion of a NOESY experiment on a system containing 16 mg (0.23 mM) porcine heart lactate dehydrogenase, 5 mM NAD^+, and 5 mM glycolate is shown in Figure 2A, and a series of time-dependent NOESY spectra corresponding to mixing times of 300, 500, 700, or 900 ms is shown in Figure 2B. The data of Figure 2B are consistent with all other cases in which ILOE peaks could be observed, and illustrate the primary difference between ILOE and TRNOE resonances: within a given ligand, there are typically many short internuclear distances, particularly corresponding to pairs of geminal or vicinal protons, while for interacting ligands, even the

closest pair of protons is likely to be $\geq$ 2.5 Å. Due to the $1/r^6$ dependence of the NOE interaction, observation of ILOE peaks typically requires the use of longer mixing times. The spectra shown in Figure 2B indicate that the glycolate binds analogously to lactate, placing the glycolate protons closest to the nicotinamide H-4 proton:



Glycolate NAD$^+$ Pyridine

Smaller cross peaks with the other nicotinamide protons are observed to increase with mixing time, while there are no cross peaks with the aromatic adenine H-2 and H-8 resonances (Figure 2A). The absence of a chiral center in glycolate makes it impossible to distinguish between interactions involving the pro-R and the pro-S hydrogen, although in principle, small shift differences will exist in the presence of the enzyme due to differences in the shifts of the two protons of the bound glycolate. More specific information on this system could in principle be obtained through the use of chirally deuterated glycolate. The above experiment was repeated on a sample containing NAD$^+$ + glycolate, but no lactate dehydrogenase. In this case, there are no ILOE peaks, and the TRNOE peaks are all positive (i.e., anti-diagonal), as expected for small molecules in the extreme narrowing limit. Hence, the observed ILOE resonances are unequivocally mediated by complexation and formation of the ternary complex.

Glucose-6-phosphate dehydrogenase from *L. mesenteroides*, a homodimer of molecular weight 108.6 kDa (Rowland et al., 1994), catalyzes the reaction:

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \rightleftharpoons$$
$$\text{NADPH} + \text{6-phosphogluconolactone}$$

NOESY spectra were obtained for the pair of reduced substrates: NADPH + glucose-6-phosphate (G6P) in the presence of the enzyme from *L. mesenteroides.* A portion of the NOESY plot showing both TRNOE and ILOE resonances for the reduced nicoti-

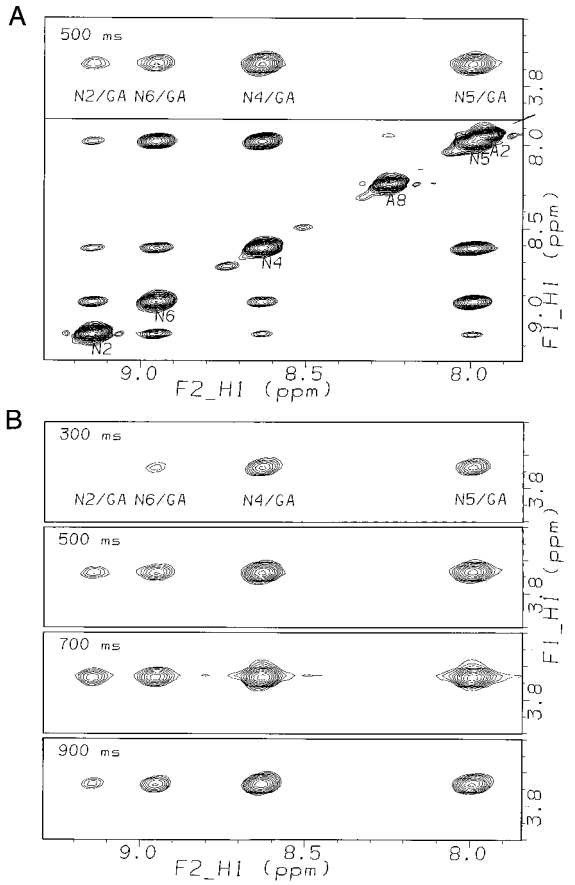


Figure 2. (A) Two-dimensional NOESY spectrum showing both the TRNOE resonances and the ILOE resonances between the glycolate ('GA') protons at δ = 3.79 ppm, and the aromatic resonances of NAD$^+$ ('N'), corresponding to a mixing time of 500 ms, in the presence of porcine heart lactate dehydrogenase. (B) ILOE peaks for mixing times of 300, 500, 700, and 900 ms, as indicated. The nicotinamide/glycolate cross peaks are labeled Ni/GA with i = 2, 4, 5, or 6 for the different nicotinamide protons. The NMR sample was prepared by dialyzing a suspension of 35.64 mg porcine heart LDH (Sigma, St. Louis, MO) against buffer (20 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM NaN_3, pH 7.4) for 24 h at 4 °C with three exchanges. After the dialysis, the H_2O buffer was exchanged with D_2O buffer (20 mM Tris-d_{11}, pH 7.4, 0.1 mM EDTA in D_2O) by centrifugation, using a centriprep-10 concentrator (10 kDa cutoff, Amicon, Beverly, MA). The concentration of the enzyme after dialysis and exchange was ~40 mg/ml. The NMR sample was made by mixing 5 mM NAD$^+$ and 5 mM glycolic acid with 0.4 ml LDH (0.23 mM). The porcine LDH is a tetramer of MW 140 kDa with 4 active sites (Bernhardt et al., 1981; Grau et al., 1981), yielding a final ratio of ligand or coenzyme to the active sites of the enzyme of 22:4. All ^{1}H NMR measurements were performed at 500 MHz on a Varian Unity-Plus 500 spectrometer at 15 °C. Phase sensitive NOESY spectra were acquired consisting of 1024 real t_2 points and 256 complex t_1 points with acquisition times of 85 ms (t_2) and 21 ms (t_1). F1 quadrature detection was achieved via the hypercomplex method. Thirty-two scans were acquired per t_1 increment to allow complete phase cycling for suppression of transverse magnetization during the mixing time, axial peaks, and F2 quadrature images. The residual HOD signal was suppressed by presaturation for 0.5 s at 0 dB power. Composite 90° pulses were used to suppress 'zz' signals (Bodenhausen et al., 1984). The total repetition time for each scan is 2.6 s, corresponding to an average accumulation time of 15 h.

namide H-4 resonances (N4A and N4B) is shown in Figure 3. Transferred NOE peaks are observed between the N2, N5, and N6 protons and the N4 protons of the nicotinamide ring, and ILOE peaks are readily observed between the nicotinamide N4A and N4B protons and the G6P H1β and H3β protons on the β anomer of glucose-6-phosphate. From these spectra, it is apparent that: (1) the NADPH + glucose-6-phosphate form a reversible, dead-end complex with the enzyme; (2) the β anomer of the glucose-6-phosphate binds much more strongly to form this ternary complex – there are no cross peaks to the α anomer, and indeed, there are only weak transferred NOE resonances within the α anomer; (3) as is particularly evident at short mixing times, the ILOE cross peak between G6P H1β and N4B is significantly larger than the cross peak between G6P H1β and N4A, indicating that the enzyme exhibits specificity for the N4B (pro-S) position. The cross peaks to both H1β and H3β imply a relative orientation of G6P and the nicotinamide ring illustrated in Figure 4A. These conclusions substantiate known features of the enzyme-catalyzed reaction. Thus, enzyme kinetic studies support the conclusion that NADPH + G6P can form a dead-end, ternary complex (Schroeder et al., 1984; H.R. Levy, personal communication). The G6P β anomer is the form known to be involved in catalysis (Salas et al., 1965; Wurster and Hess, 1974; Levy et al., 1983), and the stereochemistry of the hydride transfer is known from isotopic labeling studies to be pro-S (Arnold et al., 1976), which corresponds to N4B. Finally, the *si* face of the reduced nicotinamide ring must approach the substrate 6-phosphogluconate from the side illustrated in Figure 4B so that transfer of a proton will lead to formation of the β anomer, and this is consistent with the observation of cross peaks between the nicotinamide protons and H1β and H3β, but not H2β of the G6P in the ternary complex.

Two additional observations about the data shown in Figure 3 can also be made. First, the TRNOE cross peak between the NADPH N4 and N2 protons shows a greater intensity than the cross peaks to the N5 and N6 protons, inconsistent with the corresponding internuclear distances. In a NOESY experiment, the dependence of cross peak intensity on mixing time may be considered to be characterized by two regimes: an initial build-up regime in which the slope is related to the internuclear distance, $1/r^6$, and a decay or relaxation regime, which is dominated by the spin-lattice relaxation of the observed nucleus. As discussed above, the observation of ILOE peaks typically

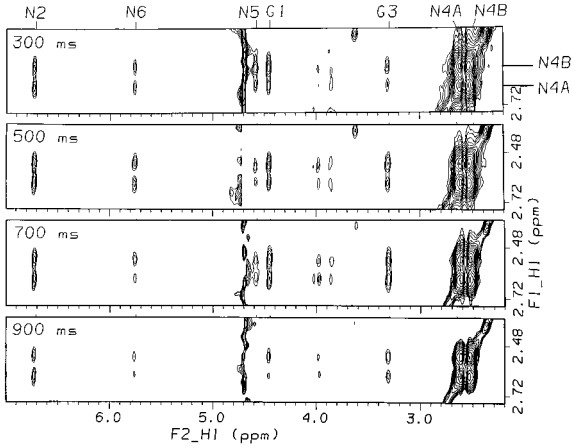


Figure 3. A portion of the two-dimensional NOESY spectrum of a sample containing 5 mM NADPH, 5 mM glucose-6-phosphate, and 0.08 mM glucose-6-phosphate dehydrogenase (homodimer, 108.6 kDa), showing the cross peaks to the nicotinamide N4A (2.62 ppm) and N4B (2.53 ppm) protons. The shift positions of the nicotinamide N2, N4A, N4B, N5, and N6 protons, and of the glucose-6-phosphate H1β ('G1') and H3β ('G3') protons are labeled as indicated. The mixing times for each of the experiments are given at the upper left corner of each spectrum. The NMR sample was prepared by dialysis of the G6PDH against protonated buffer, followed by exchange into D_2O buffer using the same procedure described above for LDH. The concentration of the enzyme after dialysis and exchange was ~9 mg/ml (0.08 mM), corresponding to a ratio of substrate or coenzyme to active enzyme sites of 62:2. To limit oxidation of NADPH to $NADP^+$, the D_2O buffer was degassed with argon, and 5 mM of sodium cyanoborohydride (Avigad, 1979) was added to the NMR sample. The experiments were set up the same way as for the LDH system (Figure 2). Two additional ILOE experiments with shorter experimental time were set up on this sample with the same pulse sequence, in order to evaluate the possible utility of the ILOE experiment for screening purposes. The first one used 16 scans per t_1 increment and reduced the experimental time by half to an average 7.5 h per experiment. The second experiment, using 128 complex t_1 points and 1024 real t_2 points with 8 scans per t_1 increment and a 1.6 s repetition time per scan, reduced the average experimental time to 1.2 h. In both of these experiments, ILOE cross peaks were clearly observed.

requires longer mixing times, since the inter-ligand distances are typically greater than many intra-ligand distances. In consequence, the mixing times selected for the data in Figure 3 correspond mostly to the build-up region of the ILOE resonances (decay is, however, evident between $\tau_m = 700$ and 900 ms), and to the decay phase for most of the nicotinamide TRNOE resonances. The longer T_1 of the N2 proton resulting from its lack of vicinal protons becomes the dominant effect, leading to a greater intensity, while N5, which has three vicinal protons, decays much more rapidly so that at the longer mixing time the TRNOE cross peak connecting N4 and N5 is lower than the ILOE peak connecting N4 and G6P H1β. A second issue relates

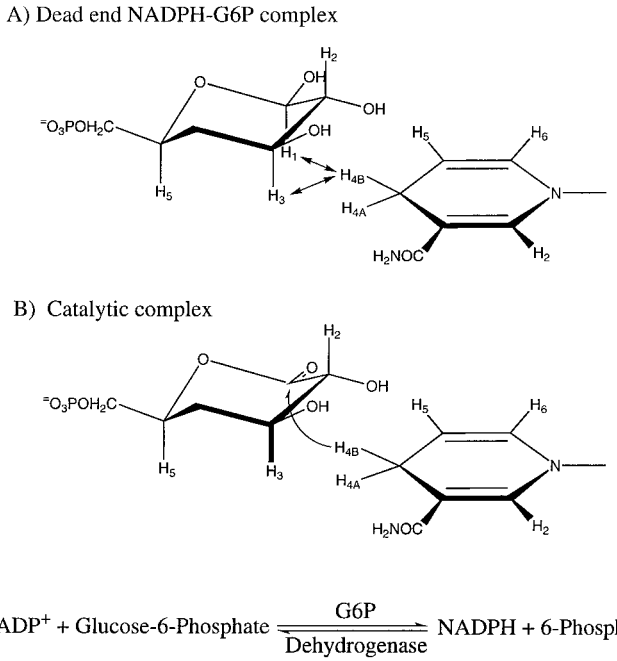


Figure 4. (A) Relative orientations of glucose-6-phosphate and the nicotinamide ring of NADPH in the ternary complex, as deduced from the data shown in Figure 3. Substituents on G6P C4 are not shown to simplify the illustration. The G6P H1β and H3β both show ILOE interactions with the nicotinamide N4 protons. (B) Relative orientation for the active complex involving NADPH and 6-phosphogluconolactone. In this relative orientation, implied by the structure of the dead-end ternary complex structure shown in A, steric conflict between the C-1 keto oxygen of the 6-phosphogluconolactone and the nicotinamide ring is avoided.

to the possibility of hydride exchange. Studies involving reduced pyridine nucleotides are limited by their susceptibility to oxidation. Small amounts of $NADP^+$ present in the sample can in principle lead to enzyme-catalyzed exchange of a hydride ion between the G6P H1β position and the pyridine N4B position. In order to limit this possibility, samples were degassed with argon and contained sodium cyanoborohydride, a mild reducing agent reported to be able to reduce pyridine nucleotides (Avigad, 1979). However, several observations suggest that this process is not that significant. Most importantly, if the ILOE cross peak connecting N4B and H1β were an exchange peak, and the other peaks were intra-residue transferred NOE peaks, then the interaction between N4B and H3β and between N4B and H5β should be about equal, since the G6P H1β-H3β and H1β-H5β distances are similar (in fact, based on galactose structural data, r(H1β-H5β) < r(H1β-H3β); Takagi, 1978; Kline et al., 1990). However, a strong peak is observed only to H3β, supporting the conclusion that this results substantially from a direct dipolar interaction between H3β and N4B. Second, older samples with more oxidation did not appear to yield more intense ILOE resonances. Finally, the direct inter-ligand NOE resonances are consistent with the existence of the NAPDH + G6P dead-end complex which is known to form (Schroeder et al., 1984).

These results, together with more detailed theoretical calculations (London, 1999), indicate that significant inter-ligand Overhauser effects in the bound state can be transferred to the uncomplexed ligands, in analogy with the transfer of conformational information in a transferred NOE experiment. Conditions for the observation of such effects are generally similar to those required for the observation of transferred NOEs, with the additional constraints that ligand affinities and concentrations must be selected such that a substantial fraction of the receptor exists as a ternary complex. For initial studies involving a search for inter-ligand Overhauser effects, relatively long mixing times are optimal. Further analysis requires time dependent data, as illustrated in Figures 2 and 3. For example, the greater proximity of NADPH N4B (relative to N4A) to the G6P H1β is most obvious at the shortest mixing times. The strong dipolar coupling between the geminal pyridine N4 protons makes evaluation of the interligand H1β-N4A and H1β-N4B distances a worst case scenario, so it is particularly

reassuring that at $\tau_m = 300$ ms significant intensity differences for the corresponding cross peaks are readily observed. Improvement in ILOE data can also be achieved by ligand design or deuteration strategies which increase the ligand T_1 values. In general, the extraction of relative inter-ligand distances is considerably more straightforward than a determination of absolute distances, as discussed in greater detail elsewhere (London, 1999). Although intra-ligand indirect dipolar relaxation effects can be readily treated within a relaxation matrix format, protein-mediated spin diffusion is a less tractable problem that can limit the accuracy of distance determinations. Such effects can in general be minimized using NOESY experiments which suppress spin-diffusion (Fejzo et al., 1992; Vincent et al., 1997) or eliminated via protein deuteration (Shibata et al., 1995). Despite this general concern, we have not observed any inconsistent or misleading ILOE cross peaks in the systems studied to date, nor have we observed any particularly misleading cross peak intensities. In a number of cases, no ILOE cross peaks were observed for ligand pairs which might have been expected to produce such effects. In most cases this appears to result from insufficient formation of the ternary complex or from sub-optimal exchange kinetics.

In general, these effects appear not to have been detected previously, probably as a consequence of the use of only a single ligand in most transferred NOE studies, or due to the need for relatively long mixing times. Two related observations have appeared in the literature involving the NAD–glutamate dehydrogenase complex (Banerjee et al., 1987) and albumin–lactate–water (Swanson, 1998), but in both cases, the interpretation of the data as arising from inter-ligand Overhauser effects is equivocal. This effect should prove valuable for the structural characterization of ternary complexes which can provide insights into active site chemistry. The ILOE approach also has potential as a mechanism for drug discovery, complementary to the SAR by NMR technique recently described (Shuker et al., 1996). For example, information analogous to that derived from Figures 3 and 4 could be used to design an inhibitor for glucose-6-phosphate dehydrogenase. In such applications, the contributions of indirect relaxation via protein-mediated dipolar coupling may facilitate the identification of ligands which bind near each other but are not in direct contact. Finally, this approach may also prove useful for the analysis of synergistic effects of environmentally significant chemicals complexed with a receptor.

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