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Distinct Heme–Substrate Interactions of Lactoperoxidase Probed by Resonance Raman Spectroscopy: Difference between Animal and Plant Peroxidases†

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ABSTRACT: Resonance Raman scattering from cow milk lactoperoxidase (LPO) and its complexes with various electron donors and inhibitors was investigated. The Raman spectrum of LPO is strikingly close to that of hog intestinal peroxidase but distinctly dissimilar to that of horseradish peroxidase (HRP). The ν_{10} frequency suggested the six-coordinate high-spin structure of heme for native LPO in contrast with the five-coordinate high-spin structure for HRP. For the ν_{10} band, benzohydroxamic acid caused a frequency shift with HRP but not with LPO. Guaiacol, *o*-toluidine, and histidine brought about a frequency shift of the ν_4 mode for LPO but not for HRP. The frequency shift was restored upon removal of the substrate or inhibitor by dialysis. The down shift of the ν_4 frequency is considered to represent an appreciable donation of electrons from the substrate or inhibitor to the porphyrin LUMO and thus their direct interaction with the heme group. From the relative intensity of the shifted and unshifted ν_4 lines, the dissociation constant was determined to be K_d = 52 mM for guaiacol and K_d = 87 mM for histidine at pH 7.4. The binding of histidine was relatively retarded in the presence of sulfate anion (K_d = 150 mM for 0.53 M sulfate present), and imidazole alone yielded no frequency shift, indicating the binding of the carboxyl group of histidine to the protein cationic site on one hand and a weak charge-transfer interaction between the imidazole group and the heme group on the other.

Lactoperoxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) is a heme-containing enzyme with a single peptide of M_r 77 000, catalyzing the oxidation of various aromatic molecules by H_2O_2 in a manner similar to plant peroxidases. The catalysis by bovine lactoperoxidase (LPO)[1] was demonstrated to proceed via the same sequential intermediates as those of horseradish peroxidase (HRP) (Kimura & Yamazaki, 1979), for which details of the catalytic mechanism have been discussed (Yamazaki et al., 1981). In contrast with a great deal of accumulated data for elucidating the interactions of the heme group with an electron acceptor (H_2O_2) and its temporal evolution during the catalytic reaction, little is known about an interaction of the peroxidase with an electron donor. The visible spectrum of the pyridine hemochrome of LPO is not of typical protoheme type (Morrison et al., 1957), but the presence of iron protoporphyrin IX was revealed through digestion of the protein by Pronase (Sievers, 1979). However, difference in the heme environments between the animal and plant peroxidases has not been fully investigated yet.

Resonance Raman (RR) spectra of peroxidase bring detailed structural information of the heme vicinity (Kitagawa & Teraoka, 1982). Combined analysis of the NMR (LaMar & deRopp, 1982) and RR data (Teraoka & Kitagawa, 1981) of HRP established the presence of a strong hydrogen bond between the proximal histidine and a surrounding amino acid residue. The strong hydrogen bond was noted to serve as a common characteristic of all plant peroxidases (J. Teraoka, D. Job, Y. Morita, and T. Kitagawa, unpublished results). We are curious to know whether such a characteristic is retained by an animal peroxidase and also to find how differently the

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[1] Abbreviations: LPO, lactoperoxidase; HRP, horseradish peroxidase; IPO, intestinal peroxidase; BHA, benzohydroxamic acid; RR, resonance Raman; LUMO, lowest unoccupied π molecular orbital.

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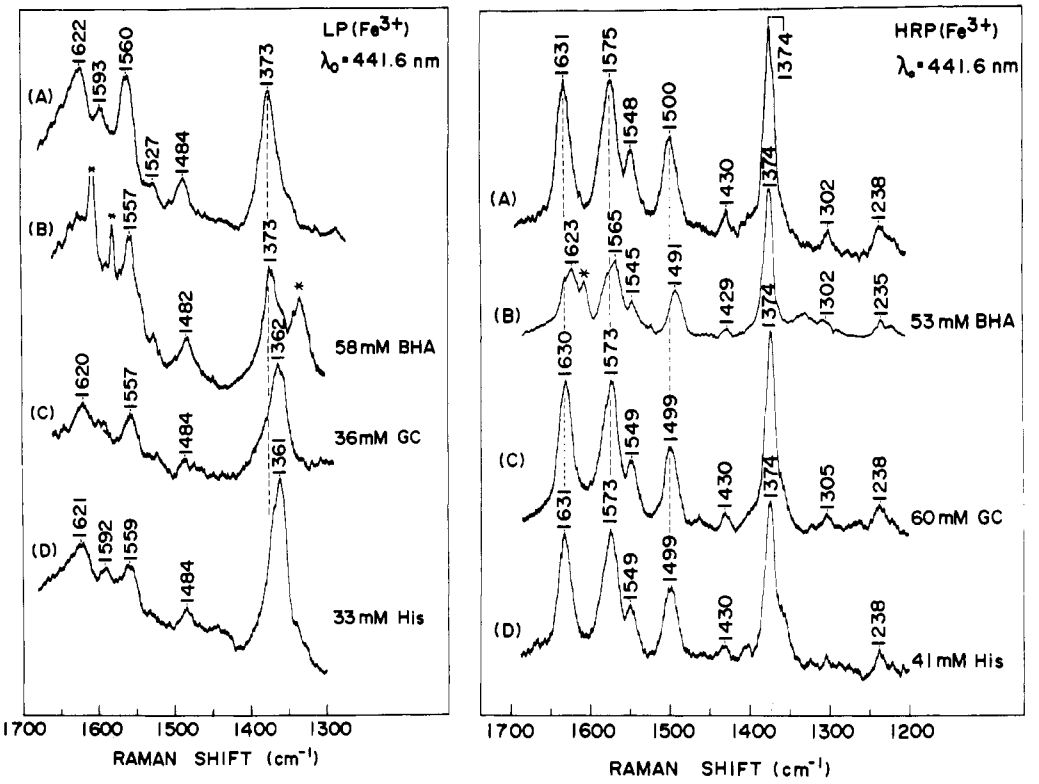

FIGURE 1: Resonance Raman spectra of bovine lactoperoxidase (left) and horseradish peroxidase (right) in the free form (A) and in the presence of benzohydroxamic acid (B), guaiacol (C), and histidine (D) (concentrations are specified at the individual spectra). The Raman lines marked by an asterisk are due to a free substrate or an inhibitor. The excitation is 441.6 nm.

electron donor interacts with the plant and animal peroxidases. Accordingly, in the present study, we investigated the RR spectra of bovine LPO and its complexes with various substrates and inhibitors in comparison with those of HRP, pointing out some distinctive features of the enzyme–substrate interactions of LPO and HRP.

Experimental Procedures

LPO was obtained from cow milk according to Rombauts et al. (1967) with slight modifications. The RZ value ($=A_{403}/A_{280}$) of this preparation was 0.83. Peroxidase activity was appraised in terms of oxidation of the electron donor and actually evaluated spectrophotometrically by following the absorbance at 470 nm of guaiacol at pH 7.4 (Hosoya & Morrison, 1967). The value for the present preparation was found to be 1.30 guaiacol units/nmol of heme. Isozyme C of HRP (RZ = 3.2) was purchased from Toyobo (grade I-C) and dissolved in a 50 mM phosphate buffer, pH 7.4.

Raman spectra were measured with the 441.6-nm line of a He/Cd laser (Kinmon Electrics, CDR80SG) and JEOL-400D Raman spectrometer. The frequency calibration was performed with indene as a standard (Hendra & Loader, 1961). For Raman measurements, 30 μL of the 100 μM LPO solution or its mixture with a substrate or an inhibitor, the concentrations of which are specified in each figure, was added to a cylindrical cell, which was placed in a water-jacketed cell holder kept at 4 °C. The inside of the Raman cell was evacuated to 0.01 Torr to avoid possible photoreaction of a substrate with oxygen, and also autoxidation in the case of the reduced enzyme.

Results

Figure 1 shows the RR spectra of oxidized LPO (left) and HRP (right) in the absence (A) and presence of a substrate (C) or an inhibitor (B and D). The RR spectra in the absence of a substrate or an inhibitor distinctly differ between LPO and HRP. The Raman lines of LPO at 1622 (dp), 1593 (p), 1588 (ap, very weak upon excitation at 441.6 nm), 1560 (dp), 1484 (p), and 1373 (p) cm^{-1} (p, polarized; dp, depolarized; ap, anomalously polarized) are assigned to the ν_{10}, ν_2, ν_{19}, ν_{11}, ν_3, and ν_4 modes of metalloporphyrin (Abe et al., 1978), although some contribution from the peripheral vinyl stretching vibration might be overlapped to the 1622-cm^{-1} band (Choi et al., 1982). The corresponding Raman lines of HRP are seen at 1631 (dp, ν_{10}), 1575 (p, ν_2), 1573 (ap, ν_{19}, very weak upon excitation at 441.6 nm), 1548 (dp, ν_{11}), 1500 (p, ν_3), and 1374 cm^{-1} (p, ν_4).

It was demonstrated previously that the frequencies of the ν_{10} and ν_3 modes are sensitive to the coordination number of the heme iron (Spiro et al., 1979; Teraoka & Kitagawa, 1980b). On the basis of the empirical rule, the five coordination of the heme iron in native HRP was pointed out from Raman spectroscopy (Teraoka & Kitagawa, 1981), in agreement with the results of the NMR (Lanir & Schejter, 1975) and ESR studies (Kobayashi et al., 1979). With the same empirical rule, the ν_{10} and ν_3 frequencies of LPO suggest the six-coordinate high-spin structure of heme, presumably with a water molecule or an alcoholic hydroxyl group at the sixth coordination position. All the plant tissue peroxidases including isozymes A_2 and C of HRP, isozymes 2, 3, 9, 15, and 16 of Japanese radish peroxidase, and isozymes 1, 3, and 7 of turnip peroxidases displayed the Raman spectra of the five-coordinate high-spin structure (Teraoka et al., unpublished results), while hog intestinal peroxidase (IPO) exhibited the Raman spectrum noticeably close to that of LPO (Kimura et al., 1981). Consequently, the dissimilarity of the iron coordination number between LPO and HRP is inferred to be one of general distinctions of the plant and animal peroxidases.

When benzohydroxamic acid (BHA) was added, no prominent RR spectral change was recognized for LPO (B in Figure 1), while the ν_{10} and ν_3 lines of HRP were shifted to 1623 and

1491 cm^{-1}, respectively, as noted previously (Teraoka & Kitagawa, 1981). The frequency shifts for HRP are suggestive of a change of the coordination number from five to six within the high-spin state. A change of the magnetic moment from 5.23 to 5.90 μ_B upon addition of BHA to HRP (Schonbaum, 1973; Maltempo et al., 1979) also implies an appreciable alteration of the heme coordination mode. For LPO, however, BHA caused presumably little structural change of the heme vicinity.

On the other hand, when guaiacol was added, the ν_4 line of LPO was shifted to 1362 cm^{-1} while the spectrum of HRP remained unaltered (C in Figure 1). Upon addition of histidine, which was found to be an inhibitor (H. Yajima and T. Hosoya, unpublished results), the very similar frequency shift took place for LPO but again not for HRP. Among other representative substrates including phenol (77 mM), pyrocatechol (110 mM), resorcinol (51 mM), *p*-hydroquinone (66 mM), and *o*-toluidine (70 mM) (numbers in parentheses denote the highest final concentration examined), only *o*-toluidine brought about a 6-cm^{-1} down shift for the ν_4 line of LPO, while others did less than 3 cm^{-1}. Addition of the same amounts of the substrates to HRP as those corresponding to the highest concentrations for the LPO experiments resulted in no shift of Raman lines, although the dissociation constants (K_d) for them in 100 mM phosphate, pH 6.0, are 7.4, 6.2, 7.9, 4.0, 3.2, and 17.6 mM for guaiacol, phenol, catechol, resorcinol, *p*-hydroquinone, and *o*-toluidine, respectively (Paul & Ohlsson, 1978). The corresponding K_d values for LPO are not reported to our knowledge.

Figure 2 illustrates the evolution of the spectral change upon addition of guaiacol to oxidized LPO. At the initial stage a shoulder appeared at the lower frequency side of the 1372-cm^{-1} band. As the shoulder grew, it seemed as if the band were gradually shifted, but actually the relative intensity of the 1372- and 1362-cm^{-1} bands was altered, and finally the shoulder dominated over the original band. When the substrate was removed through dialysis, the original RR spectrum was restored. Therefore, the two lines are considered to arise from the unbound and bound forms of the substrate, and the binding is reversible. Interestingly, other parts of the Raman spectrum are little altered by the substrate binding.

Since the two ν_4 lines are considerably overlapped, the peak height was used as a measure of the intensity. The inset figure in Figure 2 plots the ratios of the scattering intensity at 1361 and 1372 cm^{-1} against the concentrations of guaiacol (upper) and histidine (lower). On the assumption that the scattering intensities at 1372 and 1361 cm^{-1} are I_a and I_b, respectively, for LPO alone and are I_a' and I_b', respectively, for the final point of the spectral change, the midpoint concentration of the substrate, which gives K_d, corresponds to the intensity ratio of $(I_b + I_b')/(I_a + I_a')$. The K_d values thus determined are 52 mM for guaiacol and 87 mM for histidine.

The binding of histidine was affected by the presence of salts. In the same insets of Figure 2, the similar intensity ratios of the two peaks in the presence of $(NH_4)_2SO_4$ are plotted against the concentration of the substrate. The plots gave rise to K_d = 42 mM for guaiacol with 0.38 M sulfate present, and K_d = 150 mM for histidine with 0.53 M sulfate present. The K_d values obtained here may not be sufficiently precise, mainly due to systematic errors involved in the intensity estimation of Raman lines. Nonetheless, it is obvious that the binding of histidine is retarded by the presence of $(NH_4)_2SO_4$ whereas the binding of guaiacol is not. The prohibition effect was qualitatively unaltered by an exchange of the cation from NH_4^+ to Na^+. Addition of a large amount of imidazole did not yield the frequency shift of the ν_4 line. Therefore, it is likely that the carboxyl group of histidine binds to a cationic site of the protein on one hand and the imidazole group interacts weakly with the heme group on the other. A spectrophotometric determination of the binding constant was unsuccessful because of the extremely small change of absorbance upon the binding.

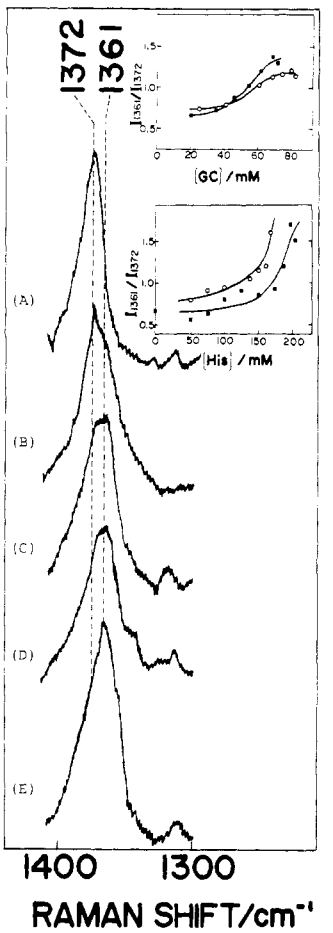

FIGURE 2: Evolution of the frequency change of the ν_4 band upon addition of guaiacol to the resting lactoperoxidase. Concentrations of guaiacol are as follows: (A) 0, (B) 24, (C) 53, (D) 62, and (E) 79 mM. The intensity of the 1362-cm^{-1} line is relatively higher than that of the 1372-cm^{-1} line, and therefore spectra D and E are recorded with an instrumental intensity one-half of others. The inset figures plot the relative scattering intensity at 1362 and 1373 cm^{-1} against the concentrations of guaiacol (upper panel) and histidine (lower panel). Open circles and closed squares denote the data obtained in the absence and presence of $(NH_4)_2SO_4$ (0.38 and 0.53 M for the upper and lower figures, respectively).

Figure 3 compares the RR spectra of the reduced forms of LPO, IPO, and HRP. The spectra of LPO and IPO are remarkably alike and are distinct from the spectra of plant peroxidases typified by the spectrum of HRP. The 1627-cm^{-1} line of HRP arises from the peripheral vinyl stretching vibration (Choi et al., 1982), but for LPO the corresponding vinyl mode is absent or overlapped to the 1617-cm^{-1} band (ν_{10}). The frequency of the ν_3 mode of LPO (1471 cm^{-1}) suggests the five-coordinate high-spin structure of the heme group similar to reduced HRP. Previously, the 244-cm^{-1} line of HRP was assigned to the Fe–His stretching vibration on the basis of the ^{54}Fe isotopic frequency shift (Teraoka & Kitagawa, 1981). Although complete identification is hard due to impracticable reconstitution of the enzyme with ^{54}Fe-incorporated heme, the 248-cm^{-1} line of LPO exhibited the characteristic pH dependence in the intensity of the Fe–His stretching Raman line (Teraoka et al., unpublished results). Accordingly,

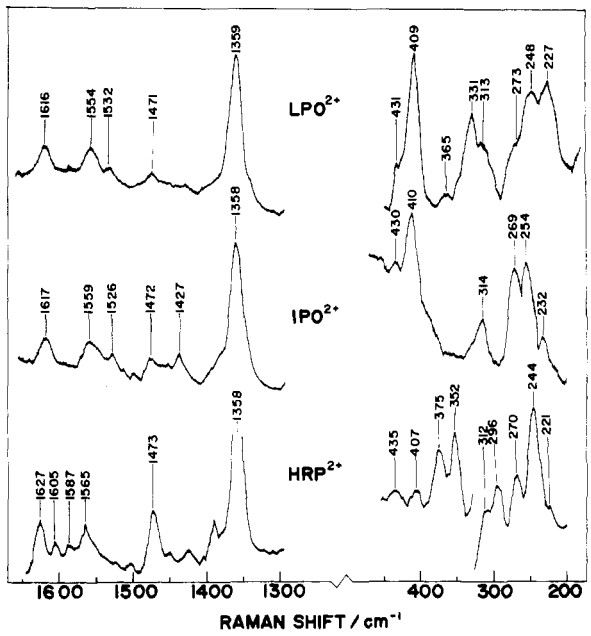

FIGURE 3: Comparison of the resonance Raman spectra of reduced lactoperoxidase (LPO^{2+}) at pH 7.4, intestinal peroxidase (IPO^{2+}) at pH 8.3, and horseradish peroxidase (HRP^{2+}) at pH 7.4, all excited at 441.6 nm. The spectra of IPO^{2+} were obtained in the previous study (Kimura et al., 1981).

the line is deduced to arise from the Fe–His stretching mode. This frequency implies the strong hydrogen bonding of the proximal histidine. Only this line is shifted to 261 cm^{-1} in the oxidized form while all other lines below 500 cm^{-1} are almost unshifted.

The ν_4 frequency of reduced LPO (1359 cm^{-1}) is noticeably close to the shifted ν_4 frequency of oxidized LPO in the presence of guaiacol or histidine. However, the frequencies of other Raman lines distinctly differ from those of the oxidized LPO–substrate or –inhibitor complexes. Furthermore, the reduction of the complexes by sodium dithionite yielded the spectrum identical with that shown at the top of Figure 3. It is therefore unlikely to assume a complete charge transfer for those complexes.

Discussion

Paul & Ohlsson (1978) studied the interaction of a number of aromatic donors with HRP by observing difference spectra and classified the donors into two groups: a group that causes a bathochromic shift of the Soret band and the other that broadens the Soret band asymmetrically with an essentially hypsochromic shift of absorption. Guaiacol and resorcinol belong to the former and *p*-hydroquinone, phenol, catechol, and *o*-toluidine belong to the latter. In the present RR spectra, however, all of these compounds did not give an appreciable shift of the ν_4 line for HRP. Therefore, the heme–ligand interaction found here is presumably of different type from those discussed by Paul and Ohlsson.

From a kinetic study, on the other hand, it was found that BHA is uncompetitive to H_2O_2 and competitive to guaiacol for LPO but conversely competitive to H_2O_2 and uncompetitive to guaiacol for HRP (H. Yajima and T. Hosoya, unpublished results). This distinct contrast between LPO and HRP is probably ascribed to different binding modes of BHA to these enzymes and is considered to be relevant to the difference in the RR spectra observed upon addition of BHA (Figure 1). The kinetic effects of histidine upon the guaiacol oxidation by HRP and LPO are now under investigation in order to see whether the RR spectral shift and kinetic information can be intimately related or not. However, it must be borne in mind that the present experiments are concerned with the free enzyme but not with the intermediates which are essential in the kinetic studies.

The ν_4 band of heme proteins appears around 1355–1362 and 1370–1375 cm^{-1} for the ferrous and ferric states, respectively, and has been postulated to reflect π delocalization of iron porphyrin (Spiro & Strekas, 1974; Kitagawa et al., 1975). This Raman line is associated mainly with the $C_\alpha N$ symmetric stretching mode (53%) and less significantly with the $C_\alpha C_m$ bending mode (21%) (Abe et al., 1978). Therefore, the frequency shift of the ν_4 line upon the binding of guaiacol, *o*-toluidine, and histidine implies the specific weakening of the $C_\alpha N$ bond upon their binding, and this must be caused by a change of π delocalization.

Recent ab initio molecular orbital calculations of iron porphyrin demonstrated that the lowest unoccupied π orbital (LUMO) of iron porphyrin is antibonding about the $C_\alpha N$ bond (Kashiwagi & Obara, 1981). Accordingly, the electron delocalization to LUMO is expected to weaken the $C_\alpha N$ bond and thus to lower its stretching frequency. In fact, when a π acidic ligand such as O_2, CO, NO, and C_2H_5NC is bound to the sixth coordination position of the ferrous heme of Mb and Hb, the ν_4 line is shifted up to ca. 1380 cm^{-1} (Spiro, 1975; Kitagawa et al., 1976) and conversely when a π donor such as RS^- is bound to the ferrous heme, the ν_4 line is shifted down to ~1340 cm^{-1} as seen for reduced cytochrome P-450 (Ozaki et al., 1976, 1978). Consequently, it is likely that guaiacol, histidine, and *o*-toluidine interact directly with the heme in the enzyme, donating some amount of electrons to LUMO.

One may argue a possibility that the binding of the particular ligand causes a conformation change of the polypeptide chain, which results in an increase of the π basicity of the fifth ligand. Indeed, the ν_4 frequency of deoxy-Hb is shifted slightly upon the conversion of the quaternary structure (Shelnutt et al., 1979), and the size of the frequency shift exhibits a linear correlation with the frequency shifts of the Fe–His stretching mode (Ondrias et al., 1982). If the similar correlation were present for LPO, a large frequency shift of Raman lines would be expected particularly below 400 cm^{-1} for the LPO–ligand complexes. However, the 261-cm^{-1} line of oxidized LPO and others as well did not display any detectable frequency shift upon the binding of guaiacol and histidine. The invariability of these Raman lines may also preclude a possibility that the particular substrate or inhibitor binds to the proximal histidine, altering its π basicity.

Previously, effects of a charge-transfer interaction between an aromatic molecule and porphyrin upon the RR spectra were examined (Shelnutt, 1981; Rousseau et al., 1982). There, small frequency shifts (0.5 to ~2 cm^{-1}) were observed for several bands including the ν_{10}, ν_3, and ν_4 lines. However, the ν_{10} and ν_3 lines are not shifted in this study. Therefore, the LPO–substrate or –inhibitor interactions observed in the present study would differ from the charge-transfer interaction discussed previously. As the RR spectra, except for the ν_4 line, are little varied by binding of the ligands, their coordination to the sixth coordination site seems less plausible. One possibility for the explanation of the present observation assumes that the ligand is stacked over the heme group with their molecular planes parallel and that nonbonding electrons of the phenoxy group of guaiacol or the nitrogen atom of histidylimidazole is directly delocalized to LUMO through a possible overlap of the two orbitals in the specific geometry.

Registry No. Peroxidase, 9003-99-0; benzohydroxamic acid, 495-18-1; guaiacol, 90-05-1; histidine, 71-00-1; phenol, 108-95-2;

catechol, 120-80-9; resorcinol, 108-46-3; *p*-hydroquinone, 123-31-9; *o*-toluidine, 95-53-4; heme, 14875-96-8; sulfate, 14808-79-8.

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Conformations of Nicotinamide Coenzymes Bound to Dehydrogenases Determined by Transferred Nuclear Overhauser Effects†

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ABSTRACT: Transferred nuclear Overhauser enhancement was used to examine the conformation of NAD^+ and $NADP^+$ bound to glucose-6-phosphate dehydrogenase and glutamate dehydrogenase and of NAD^+ bound to lactate dehydrogenase. The results demonstrate that the conformation of the nicotinamide–ribose bond is anti for dehydrogenases with A stereospecificity and syn for dehydrogenases with B stereospecificity. In those dehydrogenases that bind both NAD^+ and $NADP^+$, significant differences occur in the conformations of the bound nicotinamide coenzymes.

Dehydrogenases catalyze the stereospecific transfer of hydrogen atoms between their substrates and the coenzymes NAD or NADP. These enzymes fall into two classes, A stereospecific and B stereospecific, depending on whether the hydrogen transfer involves the *pro-R* or the *pro-S* hydrogen of the reduced nicotinamide coenzyme, respectively (You, 1982). X-ray crystallographic studies on NAD-utilizing dehydrogenases have shown that in alcohol dehydrogenase, malate dehydrogenase, and lactate dehydrogenase, all of which are A stereospecific, the conformation of the nicotinamide–ribose bond of bound NAD^+ is anti, whereas in glyceraldehyde-3-phosphate dehydrogenase, the only B-stereospecific enzyme examined, this conformation is syn (Rossmann et al., 1975) (Chart I). The NADPH-utilizing dihydrofolate re-

† From the Biological Research Laboratories, Department of Biology (H.R.L.), and the NIH Research Resource for Multi-Nuclei NMR and Data Processing, Department of Chemistry (A.E. and G.C.L.), Syracuse University, Syracuse, New York 13210. *Received March 24, 1983.* This research was supported by grants from the National Science Foundation (PCM 8102164 to H.R.L. and CHE 8105109 to G.C.L.) and the Division of Research Resources, National Institutes of Health (RR 01317 to G.C.L.).

0006-2960/83/0422-2792$01.50/0