Selectively Enriched ^{13}C-Labeled Proline-7 and ^{13}C-Labeled Leucine-8 Ocytocins as Probes for the Analysis of Peptide Hormone Interactions with Bovine Neurophysin I Using ^{13}C Nuclear Magnetic Resonance Spectroscopy†

Odile Convert, John H. Griffin, Carlo Di Bello, Pierre Nicolas, and Paul Cohen*

ABSTRACT: Ocytocin molecules containing 85% ^{13}C enrichment in the amino acid residues at positions 7 and 8 have been synthesized in order to study their interaction with bovine neurophysin I by ^{13}C nuclear magnetic resonance spectroscopy. The chemical-shift and spin-lattice relaxation time of each individual carbon atom of proline and leucine were measured on [^{13}C-Pro^7]- and [^{13}C-Leu^8]ocytocin when the hormone was free in solution or bound to neurophysin. There was no indication that these nuclear magnetic resonance parameters of the two residues are affected by hormone binding. The observed chemical shift values show that the Pro^7 peptide bond remains in the trans conformation upon complex formation. Based on the observed T_1 values, it is concluded that the segmental mobility of the entire tripeptide tail of the nonapeptide hormone is unaffected upon binding to neurophysin and that no large conformational rearrangement occurs in that region of the ligand in this process. These results together with previous spectroscopic and thermodynamic data on the binding process and with the proposed models for the preferred conformation of ocytocin in solution allow the description of a more detailed picture for the mechanism of association of this hormone to neurophysin.

The analysis of ^{13}C NMR[1] spectra of peptide hormones has contributed significantly to the understanding of the conformations of peptides (Smith et al., 1973; Deslauriers et al., 1973; Brewster et al., 1973; Walter et al., 1973) and of the molecular motion of given residues or segments of the peptides under various solution conditions (Smith et al., 1975; Deslauriers et al., 1975). Recently the use of selective ^{13}C enrichment has demonstrated the potential applicability of such an approach for sensitivity enhancement for specific assignments and for ligand binding studies (Haar et al., 1975; Griffin et al., 1975a,b, 1977; Di Bello et al., 1976). The complexes formed between the neurohypophyseal protein, neurophysin, and the neurohypophyseal hormone ocytocin are particularly well-suited for such ^{13}C NMR analyses since considerable information is now available on the thermodynamics (Camier et al., 1973; Cohen et al., 1975; Breslow, 1975), kinetics (Alazard et al., 1974; Pearlmutter and McMains, 1977), and stereoselectivity of the binding reaction (Nicolas et al., 1976, 1977). The important influence of hormone binding on the dimerization of neurophysin monomers of 10^4 molecular weight has been recently clarified (Nicolas et al., 1976). The use of tripeptide analogues of the N-terminal sequence of the neurohypophyseal hormones (Breslow, 1975) together with CD and absorbance spectroscopic analyses (Griffin et al., 1973; Cohen et al., 1975) and proton NMR studies (Alazard et al., 1974; Balaram et al., 1973) have clearly demonstrated the critical contribution of residues 2 and 3 of the hormones in the binding reaction. These conclusions about residues 2 and 3 were also supported by ^{13}C relaxation times measurements suggesting the restricted mobility of the side chain of residue 3 in the complexed hormone (Griffin et al., 1975a; Di Bello et al., 1976) compared with the unaltered motional freedom of residue 9 of the C-terminal tail (Griffin et al., 1975b, 1977). However, the observed difference in behavior between ocytocin and vasopressin (Camier et al., 1973; Nicolas et al., 1976) together with the known difference in binding affinity of the N-terminal tripeptide analogues vs. the nonapeptide hormones have raised the question of the possible involvement of other amino acid residues in complex formation. The synthesis of selectively ^{13}C-enriched derivatives of ocytocins labeled in Pro^7 and Leu^8 allowed the study reported here of the segmental and local mobility of the entire tripeptide C-terminal tail in the free and neurophysin-bound molecules.

Materials and Methods

Uniformly labeled 85% ^{13}C-enriched leucine and proline were prepared at the Service des Molécules Marquées (Centre d'Etudes Nucléaires, Saclay) as described (Tran-Dinh et al., 1974). The synthesis of the nonapeptide ocytocin was made using the solid phase method (Merrifield, 1965) with minor modifications (Griffin et al., 1975b). The cleavage of the peptide from the resin, HF deblockage, and ferricyanide oxidation have been described (Griffin et al., 1975b). The purification of the crude peptide was performed on Sephadex G-15 columns according to Manning et al. (1970). In all cases a final additional purification step was necessary in order to separate ocytocin from minor contaminants. This was performed as already described (Griffin et al., 1977). The purity of each

† From the Laboratoire de Chimie Organique Structurale, Université P. et M. Curie, Paris, France (O.C.), The Scripps Clinic and Research Foundation, La Jolla, California 92037 (J.H.G.), Istituto di Chimica Generale, Università di Padova, 35100 Padova, Italy (C.D.B.), and the Groupe de Neurobiochimie Cellulaire et Moléculaire, Université P. et M. Curie, 96 Boulevard Raspail, 75006 Paris, France (P.N. and P.C.). *Received June 2, 1977.* Supported in part by grants from the Université P. et M. Curie, from the Centre National de la Recherche Scientifique to J.H.G. and C.D.B. as visitors, and to P.C. (Recherches Coopératives sur Programme 220 and Equipe de Recherches Associée 693) and from NATO (1007) to P.C. and C.D.B. J.H.G. is the recipient of a National Institutes of Health Research Career Development Award. This is publication number 1372 of the Department of Immunopathology of the Scripps Clinic and Research Foundation, La Jolla, California 92037.

[1] The nomenclature and abbreviations used in this paper follow the rules and recommendations of the IUPAC-IUB Commissions on Biochemical Nomenclature; see for instance: (1972), *Eur. J. Biochem. 27,* 201-207; (1975), *Eur. J. Biochem. 55,* 485-486. Other abbreviations are: NMR, nuclear magnetic resonance; UV, ultraviolet; CD, circular dichroism.

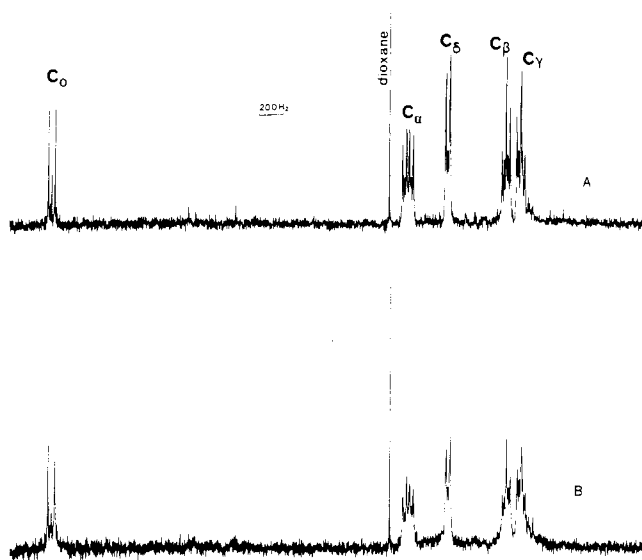

FIGURE 1: ^{13}C NMR spectra of 85% ^{13}C-enriched Pro[7] ocytocin. (A) Peptide is 7 mM (91 000 transients); (B) same sample + 0.28 equiv of neurophysin I (91 000 transients).

TABLE I: Chemical Shift Values for 85% ^{13}C-Enriched Pro[7] and Leu[8] Ocytocins Given in ppm Downfield from Me_4Si.[a]

Carbon atom	Ocytocin (7 mM) alone at pH 7.0	Ocytocin (7 mM) + 0.28 equiv of neurophysin, pH 7.0
Pro[7] C_O	175.22	175.23
Leu[8] C_O	176.14	176.15
Pro[7] C_α (CH)	61.52	61.47
Leu[8] C_α (CH)	53.47	53.49
Pro[7] C_β (CH_2)	30.11	30.07
Leu[8] C_β (CH_2)	40.06	40.07
Pro[7] C_γ (CH_2)	25.38	25.32
Leu[8] C_γ (CH)	25.38	25.40
Pro[7] C_δ (CH_2)	48.76	48.71
Leu[8] C_{δ_1} (CH_3)	22.77	22.80
Leu[8] C_{δ_2} (CH_3)	21.35	21.39

[a] The chemical shift values were taken as the center of the observed multiplets when deviation from first order was observed.

preparation of ocytocin was assessed by thin-layer chromatography on cellulose plates run in two different solvent systems. Each peptide yielded a single spot upon staining with ninhydrin or a disulfide stain (Griffin et al., 1975b). The amino acid composition of each sample was determined following acid hydrolysis on a Carlo Erba 3A28 automatic amino acid analyzer. Each peptide gave the expected theoretical values within ±10%. The ability of each peptide to bind neurophysin was checked using the UV absorbance difference spectroscopy based on the perturbations due to binding observed in the 240–320-nm region (Griffin et al., 1973). Positive absorbance differences at 287 nm with $\Delta\epsilon$ of 400 and 420 $M^{-1}\ cm^{-1}$ were measured (on a Cary 118C spectrophotometer) respectively with the Pro[7]- and Leu[8]-^{13}C-enriched ocytocins in excellent agreement with the usual amplitude recorded using reference ocytocin in the standard conditions (Griffin et al., 1973; Wolff et al., 1975). Each [^{13}C]ocytocin was tested for its pharmacological activity and compared with reference ocytocin (Sandoz, Basle). In the pig kidney medulla adenylate cyclase assay (Pradelles et al., 1972) and in assays of the insulin-like activity of ocytocin on glucose oxidation by isolated rat adipocytes (Bonne and Cohen, 1975), each ^{13}C-enriched ocytocin gave full biological activity (±10%). Bovine neurophysin I was purified by isoelectric focusing as previously described (Camier et al., 1973) and its purity was routinely checked by slab gel electrophoresis. ^{13}C NMR spectra were recorded on a Varian XL 100-12 spectrometer operating in Fourier transform mode at 25.15 MHz interfaced with a Varian 620 L computer. For a spectral width of 5000 Hz, an acquisition time of 0.8 s was used, giving a resolution of 1.25 Hz. Normal spectra were obtained with complete proton decoupling from a 1.3-mL sample of 7 mM peptide solutions in D_2O, 0.1 M NaCl, adjusted at pH 7.0 (uncorrected meter reading). pD values should be considered as pH read on the meter +0.4 unit. D_2O provided an internal deuterium lock signal. Five microliters of dioxane was added to the sample to provide the internal reference peak. Chemical shifts values are expressed downfield from tetramethylsilane (Me_4Si) assuming the dioxane peak is 67.4 ppm downfield from external Me_4Si. Spin–lattice relaxation measurements were performed by the inversion recovery method (Freeman and Hill, 1970) using a pulse sequence (180°-τ-90°-T) where τ is a variable delay time and T is at least five times longer than the longest T_1 to be measured. The width of the 90° pulse was 16 ms. T_1 values were determined by a least-squares fit of a semilogarithmic plot of the best straight line. The accuracy of the T_1 values is ±15%. After the ^{13}C NMR spectra and T_1 measurements on hormone solutions had been performed, a weighed amount of dry lyophilized neurophysin was added to the peptide solution and stirred magnetically while microliter amounts of 1 N NaOH were added to maintain the pH around 7.0. No precipitation of the samples was observed under these experimental conditions. After the spectra had been run, and the T_1 values determined, the protein and peptides were separated on a Sephadex G-25 column in HCOOH (0.1 N) and the separately recovered compounds were found apparently unaltered as judged on thin-layer chromatography plates for the peptides and on polyacrylamide slab gel electrophoresis for the neurophysin.

Results

[^{13}*C-Pro*[7]]*Ocytocin.* Figures 1A and 1B show the spectra of the 85% enriched proline residue of ocytocin in the absence or in the presence of 0.28 equiv of bovine neurophysin I. These spectra exhibited only the set of signals corresponding to each carbon atom of the enriched residue with no appearance of individual peaks from other atoms either of the peptide or of the added protein. Complications in the spectra arising from the presence of other ^{13}C atoms which give rise to ^{13}C one, two, and three bond ^{13}C–^{13}C coupling were apparent. They were similar to those observed for the amino acid alone (Fermandjian et al., 1975). In the 1.0 to 30 ppm portion of the spectrum, deviation from first order was observed (Figure 1). Despite these complexities, the 85% enrichment allowed a good measurement of the chemical shift value for each carbon atom of the proline residue in ocytocin (Table I). Addition of 0.28 equiv of neurophysin resulted in no observable change of the measured chemical shifts (Table I). Only a slight broadening of the lines, resulting from complex formation, was observed. It has been shown that the peptide in solution is in rapid exchange at the NMR time scale between the bound and free states (Alazard et al., 1974). In this case the observed NMR signals are the weighed average of the values for the bound and free ocytocin (Cohen et al., 1972; Alazard et al., 1974). In contrast to many other peptides where cis-trans isomerism around the peptide bond of proline has been observed (Haar et al., 1975; Deslauriers et al., 1973), there is no evidence in this spectrum for lines indicating the coexistence of two conformers, and the

TABLE II: NT_1 Values in Milliseconds (±15%) for ^{13}C Atoms of Pro^7 and Leu^8 Ocytocins.[a]

Carbon atom	Ocytocin (7 mM) alone	Ocytocin (7 mM) + 0.28 equiv of neurophysin I
Pro^7 C_α (CH)	143	127
Leu^8 C_α (CH)	167	159
Pro^7 C_β (CH_2)	286	264
Leu^8 C_β (CH_2)	252	254
Pro^7 C_γ (CH_2)	304	290
Leu^8 C_γ (CH)	300	309
Pro^7 C_δ (CH_2)	178	144
Leu^8 C_{δ_1} (CH_3)	1340	1164
Leu^8 C_{δ_2} (CH_3)	1668	1612

[a] The given value of NT_1 is the mean value obtained from analysis of each signal of the multiplets arising from ^{13}C–^{13}C coupling.

chemical shift values of the carbon atoms of proline 7 in ocytocin (Table I) are those typical of the trans peptide bond configuration (Brewster et al., 1973).

To assess the relative mobility of each one of the four carbon atoms of the pyrrolidine ring of the Pro^7 residue, measurements of the spin–lattice relaxation time T_1 were performed (Table II). Previous work has clearly indicated the value of such determinations to obtain information about the segmental or local motional freedom of carbon atoms in peptide molecules (Smith et al., 1975). For small peptides undergoing rapid, isotropic motion in solution, in the extreme narrowing limit, the longer the observed NT_1 value (where N is the number of directly bound protons of the ^{13}C nuclei) the greater is the mobility of the considered carbon atom. This was also studied in the case of ocytocin using natural abundance ^{13}C NMR spectra (Deslauriers et al., 1974). Table II shows the NT_1 values obtained for each of the proline carbon atoms of ocytocin either free or in the presence of 0.28 equiv of neurophysin II. No drastic modification of the relaxation times was measured consecutive to protein addition. Only a slight possible decrease of 5 to 20% was observed affecting all the considered atoms with the largest effect on the C_δ atom. As previously reported (Deslauriers et al., 1974) for ocytocin at the natural abundance, the mobility of the pyrrolidine ring is detectable only at the β and γ carbons. The δ carbon is nearly as restricted as the α carbon, due to attachment of the bulky cystine 6 residue to the imino nitrogen of proline.

[^{13}C-Leu^8]Ocytocin. The spectrum of the synthesized ocytocin containing 85% enriched [^{13}C]leucine at position 8 is shown in Figure 2A. The spectrum resembles that of the free amino acid and that of the 85% ^{13}C-enriched Leu^3-ocytocin (Griffin et al., 1977). Table I summarizes the values for the chemical shifts of each carbon atom. It shows that addition of 0.28 equiv of neurophysin I had no effect on the chemical shifts values of each one of the five carbon atoms of the leucine side chain. The NT_1 values (Table II) obtained for the C_β and C_γ atoms were comparable in magnitude to the one measured on the corresponding atoms of the pyrrolidine ring of proline. But the NT_1 value for the C_α was greater in the case of leucine compared with proline probably as a consequence of increased mobility toward the C terminus portion of the peptide. The large C_{δ_1} and C_{δ_2} NT_1 values (1.4 to 1.7 s) reflected the high degree of freedom of the terminal atoms of this aliphatic side chain. In this case, addition of 0.28 equiv of neurophysin I produced no effect, within experimental error (±15%), on each of the NT_1 values (Table II).

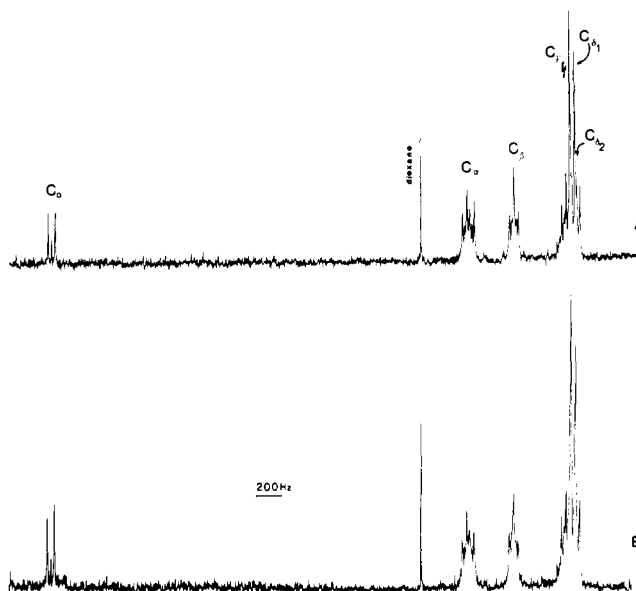

FIGURE 2: ^{13}C NMR spectra of 85% ^{13}C-enriched Leu^8 ocytocin. (A) Peptide is 7 mM (50 000 transients); (B) same sample + 0.28 equiv of neurophysin I (50 000 transients).

Discussion

The studies on [^{13}C-Pro^7]- and [^{13}C-Leu^8]ocytocins indicate clearly the value of isotopic enrichment to enhance the sensitivity of ^{13}C NMR spectroscopic analysis of peptide hormone. The observations made here permit definitive assignments of the resonances of these two residues as previously observed in natural abundance studies by various authors (Brewster et al., 1973; Walter et al., 1973, 1974). The existence of signals reflecting the presence of a unique conformer around the pyrrolidine ring shows that both in the free and neurophysin bound states this peptide is entirely in the trans form. The fact that the trans isomer population was not affected by neurophysin binding suggests strongly that no large conformational rearrangement of the tripeptide tail of the ligand occurred upon hormone binding.

The choice of selective enrichment at two amino acid residues of the C-terminal-exocyclic portion of the hormone molecule allows us to gain information on the segmental and local mobility of this portion of the peptide in the free and the bound states. The measured relaxation times values for the various carbon atoms were in the same ratios as those reported by Deslauriers et al., although they were, in general, longer than the reported values (Walter et al., 1974). Since they were obtained under different concentration (7 mM vs. 100 mM) and pH conditions (pH 7.0 vs. 3.5), the systematic differences in T_1 may reflect the fact that a 15-fold increase in concentration may result in a significant increase of the solution viscosity accompanied by a decrease in the overall motion of the peptide molecule.

Addition of neurophysin to the hormone solution resulted in rather small if any decreases of the NT_1 values of the proline carbons. The discrete effect observed is compatible with the conclusion that residue 7 of ocytocin is not directly involved in an interaction with the neurophysin binding sites.

The interpretation of the observed NT_1 values for [^{13}C-Leu^8]ocytocin is straightforward. The data show that the carbon atoms of the aliphatic side chain possess an increasing mobility from the C_α to the C_δ and that this freedom is unaltered by hormone binding to the neurohypophyseal protein. This supports the conclusion that none of the carbon atoms of residue 8 of ocytocin is engaged in a direct interaction with

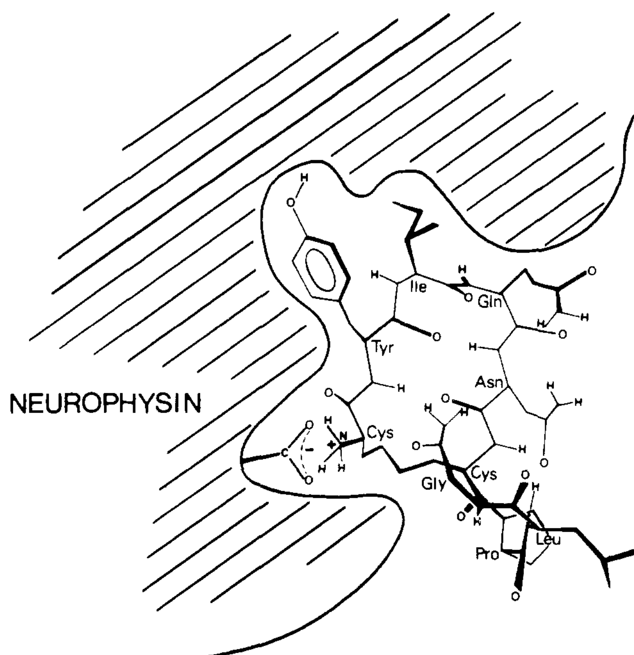

FIGURE 3: A possible schematic representation of ocytocin binding to the high affinity binding site of a neurophysin protomer on the dimeric molecule. The parallel diagonal lines in the neurophysin are meant to indicate a hydrophobic region of the protein molecule. The peptide backbone of ocytocin has been represented. Its N-terminal tripeptide portion is presumed to be involved in interactions with the site while the C-terminal tripeptide tail is represented free from any direct bonds with the protein (see text for discussion)

neurophysin and that the leucine side chain remains mobile in the hormone-protein complex. In this context, it is important to note that the observed NT_1 values for the δ carbons of [^{13}C-Leu^3]ocytocin decreased from 1.7 to 0.58 s in similar experiments performed with the ^{13}C enrichment at position 3 of ocytocin (Griffin et al., 1977).

The results collected from both proton and ^{13}C relaxation time measurements have shown that, in the neurophysin-ocytocin complex, residues 2 and 3 of the hormone are restricted in their motions (Alazard et al., 1974; Griffin et al., 1977), while residues 9 (Griffin et al., 1976, 1977), 7, and 8 (vide supra) remain free of motional restrictions by intermolecular interactions. Taken together with other data available on the binding process (see reviews by Cohen et al., 1975; Breslow, 1975) and on ocytocin conformation in aqueous solutions (Walter et al., 1971; Glickson et al., 1976), the new ^{13}C NMR results allow us to give a more detailed picture for the association of ocytocin to neurophysin than that previously proposed (Griffin et al., 1973; Cohen et al., 1975). In Figure 3 the following features have been schematically represented.

(i) The overall conformation of ocytocin is that proposed by Urry and Walter (1971).

(ii) The side chains of residues 2 and 3 are rigidly bound in a hydrophobic pocket of the neurophysin site.

(iii) An electrostatic interaction is indicated between a carboxyl group of the protein and the NH_3^+ of the N terminus of the hormone molecule.

(iv) Residues 7, 8, and 9 of the tripeptide tail are free of any interactions with the protein and the conformation of the peptide bond of proline remains in the trans form in the complex.

(v) This represents the association of one ocytocin molecule to the high affinity binding site of each protomer of a neurophysin dimer (Nicolas et al., 1976). It should be emphasized that the schematic model in Figure 3 is a simplistic model in many respects. For example, no consideration is made for neurophysin dimerization, for the second, weaker, binding site, or for other sites of interaction, such as specific hydrogen bonding, between ocytocin and neurophysin. Also there is evidence both theoretical and experimental suggesting conformational flexibility for ocytocin in aqueous and other solutions (Kotelchuck et al., 1972; Brewster et al., 1973; Meraldi et al., 1977; Glasel et al., 1973). Nonetheless, this model accounts for a large amount of spectroscopic and thermodynamic data and it is useful for the design of future experimental investigations.

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Convergence of Active Center Geometries†

R. Michael Garavito,‡ Michael G. Rossmann,* Patrick Argos, and William Eventoff§

ABSTRACT: Comparisons have been made between the active center geometries of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, chymotrypsin and papain, and glyceraldehyde-3-phosphate dehydrogenase and papain. In the dehydrogenases, orientation of the nicotinamide ring about the glycosidic bond is determined by the substrate stereochemistry. The proper positioning of the carboxyamide moiety allows for the close approach of the C4 atom on the nicotinamide and the reactive carbon of the substrate. It follows that, once the conformation of the substrate or substrate intermediate has been established with respect to the functional groups in the enzyme, the A- or B-side specificity of the nicotinamide ring is predetermined. Hence, dehydrogenases which are divergently evolving from a common precursor must maintain the nicotinamide specificity if the protein fold of the catalytic domain is conserved. The tetrahedral intermediates produced during acylation of chymotrypsin and papain are found to be of opposite hand, while those of papain and glyceraldehyde-3-phosphate dehydrogenase can be regarded to be of the same hand. Thus the serine proteases, subtilisin and those of the chymotrypsin family, are of one hand while the cysteine enzymes, glyceraldehyde-3-phosphate dehydrogenase and papain, are of the other.

The similarity of the active centers of subtilisin and chymotrypsin (Kraut et al., 1971) is the prime example of convergence of active center geometries in enzymes, although Drenth et al. (1976a) and Polgár (1977) have recently commented on further possible similarities of catalytic sites. While chymotrypsin and subtilisin have totally different foldings of their polypeptide backbones, the residues involved in catalysis (serine, histidine, aspartic acid) have the same spatial relationships. Superposition of the atoms in the active centers shows a root-mean-square difference of only 1.0 Å (Kraut et al., 1971).

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§ Present address: SDC Integrated Services, 13 Kroeger Executive Center, Norfolk, Virginia 23502.

It will be shown, by comparing various active center regions, that convergence is a common occurrence. The selected enzymes were specific for a substrate with a carbonyl group which undergoes a trigonal–tetrahedral carbon transition during catalysis. Such substrates and the respective active center moieties will mutually impose chemical and geometrical constraints on the reaction mechanisms. Thus, a comparison of analogous enzymes can determine the essential features of catalysis. Furthermore, the interrelationship of the chirality of the substrate and coenzyme for dehydrogenases can be established.

Experimental Section

A computer program was written based on the superposition techniques of Rao and Rossmann (1973) and Rossmann and Argos (1975). The program was informed of the atoms to be equivalenced which then provided an initial rotation matrix and translation vector. The Eulerian angles and translational components were refined to provide the best weighted least-squares fit between equivalenced atoms. Atoms separated by