Interaction of Enkephalins and Des-tyrosyl-enkephalins with Synaptosomal Plasma Membrane Vesicles: Enkephalin Binding and Inhibition of Proline Transport†

D. E. Rhoads,‡ H. Sankaran,§ N. A. Peterson, and E. Raghupathy*

Department of Psychiatry, Brain-Behavior Research Center, Sonoma Developmental Center, University of California, San Francisco, Eldridge, California 95431

Received August 23, 1985

ABSTRACT: Leucine- and methionine-enkephalins inhibit the Na^+-dependent transport of proline into plasma membrane vesicles derived from synaptosomes. Glycine transport is weakly inhibited by enkephalins whereas there is no inhibition of transport of glutamic acid, aspartic acid, or γ-aminobutyric acid. The inhibition of proline uptake is observed with des-tyrosyl-enkephalins but not with morphine, dynorphin(1–13), or β-endorphins. Furthermore, enkephalin-induced inhibition of proline transport is not antagonized by naloxone. [Leu]enkephalinamide and modified [Leu]enkephalins with greater selectivity for the δ-subclass of enkephalin binding sites are less effective than [Leu]enkephalin in the inhibition of proline transport. Specific binding of [^{3}H]Leu-enkephalin to the plasma membrane vesicles is demonstrated, and des-Tyr-[Leu]enkephalin competes with Leu-enkephalin for [Leu]enkephalin binding sites. The similarity in the concentrations of des-Tyr-[Leu]enkephalin required to compete for specific [Leu]enkephalin binding and to inhibit proline transport suggests that a specific subclass of enkephalin binding sites, distinguished by their recognition of both the enkephalins and their des-tyrosyl derivatives, may be associated with the synaptic proline transport system.

The opioid pentapeptides leucine-enkephalin ([Leu]enkephalin) and methionine-enkephalin ([Met]enkephalin) have a variety of effects on the electrophysiology of neurons of the mammalian central nervous system [reviewed by Nicoll (1982)]. These effects appear most often as an inhibition of neuronal activity and could result from pre- or postsynaptic actions of the peptides. The presynaptic membrane is involved in both the release and uptake of amino acids, many of which are putative neurotransmitters. Presynaptic release of neurotransmitters has been shown to be inhibited by opiates and opioid peptides (Mudge et al., 1979). This action was ascribed to the inhibition of Ca^{2+} mobilization or Ca^{2+} entry into the presynaptic nerve terminal, thereby preventing the Ca^{2+}-dependent exocytotic release of neurotransmitters. Another presynaptic function of nerve terminals involves the reuptake of released neurotransmitters, especially amino acid neurotransmitters, via high-affinity, Na^+-dependent transport systems (Iversen, 1971). We recently reported (Rhoads et al., 1983b) that the high-affinity, Na^+-dependent transport system for proline in isolated nerve terminals (synaptosomes) prepared from rat cerebral cortex was inhibited by enkephalins. In contrast, the uptake of amino acid neurotransmitters such as γ-aminobutyric acid and glutamic acid was not altered in the presence of enkephalins.

A variety of electrophysiological studies [reviewed by Giacobini (1983)] has demonstrated the potential neuroactivity of proline and led to the suggestion that proline may have a role as an inhibitory neurotransmitter in the mammalian central nervous system. The properties of the uptake and release of proline in brain slices, synaptosomes, and plasma membrane vesicles derived from synaptosomes are similar to those of known neurotransmitter amino acids (Peterson & Raghupathy, 1972; Bennett et al., 1972; Balcar & Johnston, 1974; Mulder & Snyder, 1974; Balcar et al., 1976; Rhoads et al., 1982a–c). Inhibition of synaptosomal proline uptake by enkephalins was not blocked by the classical opioid antagonist naloxone, and the des-tyrosyl derivatives of the enkephalins inhibited to the same degree as the intact pentapeptides (Rhoads et al., 1983b). These results suggested that the inhibition of proline uptake is due to a nonopioid property of the enkephalins and may, therefore, be mediated by a new subclass of enkephalin receptor, perhaps uniquely coupled to the proline carrier.

In the present study, we employ a membrane vesicle preparation to further investigate the interaction of enkephalins with the synaptic proline carrier and describe the presence of an enkephalin-sensitive proline transport system in plasma membrane vesicles derived from synaptosomes. The specificity of the inhibition with regard to the enkephalins and the amino acid transport systems is consistent with results obtained with intact synaptosomes. Specific binding of [^{3}H]Leu-enkephalin to this membrane preparation reveals a direct correlation between the concentration curves for the inhibition of proline uptake by des-Tyr-[Leu]enkephalin and displacement of [^{3}H]Leu-enkephalin binding by des-Tyr-[Leu]enkephalin.

Experimental Procedures

Materials. L-[U-^{14}C]Amino acids were purchased from New England Nuclear Corp. (Boston, MA) and had the following specific activities (mCi/mmol): proline, 293; aspartic acid, 219; glutamic acid, 271, glycine, 104, γ-aminobutyric acid, 213. [^{3}H]Leucine-enkephalin (50 Ci/mmol) was also obtained from New England Nuclear Corp. The enkephalins and β-endorphins were purchased from Sigma Chemical Co. (St. Louis, MO) and Bachem (Torrance, CA). Naloxone and dynorphin(1–13) were obtained from Dr. Horace Loh (Department of Pharmacology, University of California at San

†This research was supported by Grant NS-15659 from the U.S. Public Health Service and Grant 82N-38 from the American Heart Association, California affiliate.

‡California Heart Association research fellow. Present address: Department of Biochemistry and Biophysics, University of Rhode Island, Kingston, RI 02881.

§Surgical Service, V.A. Medical Center, San Francisco, CA 94121.

0006-2960/86/0425-1580$01.50/0

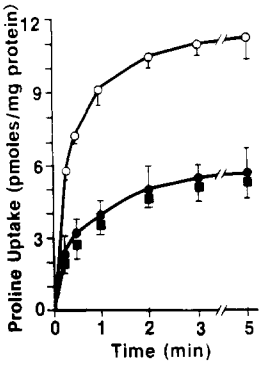

FIGURE 1: Time course of vesicular proline transport and its inhibition by [Leu]enkephalin in the presence and absence of naloxone. Accumulation of proline into plasma membrane vesicles derived from synaptosomes was determined after various time intervals in the presence (●) and absence (○) of 15 μM [Leu]enkephalin and also in the presence of 15 μM [Leu]enkephalin and 150 μM naloxone (■). The values are the means ± SEM of three to five independent determinations.

Francisco). Membrane filters (0.45 μm) were obtained from Micro Filtration Systems (Dublin, CA).

Methods. Procedures used for preparing synaptosomal plasma membrane vesicles, loading the vesicles, and determining amino acid transport were as described previously (Rhoads et al., 1982a). The vesicles were loaded with 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM $MgSO_4$. The loaded vesicles (0.1–0.2 mg of protein) were incubated with 0.1–0.2 μCi of [^{14}C]amino acid in 1 mL of buffer A consisting of 150 mM NaCl, 1 mM $MgSO_4$, and 5 mM Tris-HCl,[1] pH 7.4. Incubations were terminated after the appropriate time periods by membrane filtration as described previously (Rhoads et al., 1982a). Under these or similar conditions, ion concentration gradients are artificially established across the vesicle membrane and provide the driving force for concentrative, high-affinity uptake of glutamic acid (Kanner & Sharon, 1978), γ-aminobutyric acid (Kanner, 1978), glycine (Mayor et al., 1981), aspartic acid (Marvizon et al., 1981), and proline (Rhoads et al., 1982a).

Enkephalin binding studies were performed as described by Chang et al. (1983) except that [^{3}H]Leu-enkephalin was employed as the tracer ligand. This required that bacitracin (100 μg/mL) be added to the incubation mixtures to retard proteolytic degradation of the [^{3}H]Leu-enkephalin. Binding was determined in buffer A or in buffer B (Rhoads et al., 1983b) containing 130 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EGTA, and 10 mM MOPS, pH 7.4. This latter buffer was routinely used in synaptosomal amino acid transport studies. Incubations were terminated by membrane filtration. Nonspecific binding was taken as the radioactivity associated with the filtered vesicles after incubation of the vesicles in the presence of a 1000-fold excess of unlabeled over radiolabeled [Leu]enkephalin. Values for the total enkephalin binding were corrected for this nonspecific binding; hence, all values reported represent specific enkephalin binding by this criterion. The results of binding studies performed in buffer A or B were not significantly different.

RESULTS

Amino Acid Uptake. Time courses for proline uptake into the synaptosomal plasma membrane vesicles and for the inhibition of proline uptake by [Leu]enkephalin are illustrated in Figure 1. In each case, maximal proline accumulation was attained after 2–3 min. In the presence of 15 μM [Leu]-enkephalin, the overall capacity for proline accumulation was reduced by approximately 50%, as was the initial rate of proline uptake. Also shown in Figure 1 is the lack of effect of naloxone on the inhibition of proline uptake by [Leu]-enkephalin. In the experiments illustrated, naloxone was present in a concentration that was 10-fold greater than the concentration of [Leu]enkephalin. Lack of antagonism by naloxone was also observed in experiments where the membrane vesicles were preincubated with naloxone prior to the addition of [Leu]enkephalin and the start of the transport assay. Naloxone alone had no effect on proline uptake.

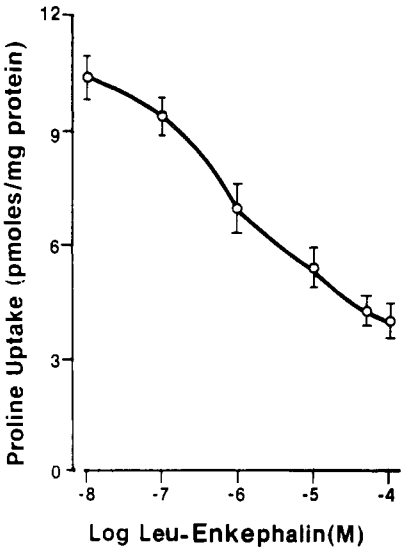

FIGURE 2: Dose dependence of the inhibition of proline uptake by [Leu]enkephalin. Proline accumulation was determined after 3-min incubations with varying concentrations of [Leu]enkephalin. The values are the means ± SEM of five independent determinations.

Table I: Effects of Morphine and Opioid Peptides and Their Derivatives on Proline Accumulation by Plasma Membrane Vesicles Derived from Synaptosomes

addition (15 μM)	proline accumulation (% of control)[a]
none (control)	100
[Leu]enkephalin	45 ± 5
des-Tyr-[Leu]enkephalin	43 ± 10
[Met]enkephalin	50 ± 7
des-Tyr-[Met]enkephalin	52 ± 8
morphine	94 ± 6
dynorphin(1–13)	93 ± 9
β_n-endorphin	99 ± 3
[Leu^5]-β-endorphin	96 ± 8
[Leu]enkephalinamide	73 ± 9
DSLET	89 ± 6
DADLE	96 ± 7

[a] Proline accumulation was determined after 3 min of incubation in the presence or absence of the additions listed. Experimental values are expressed as the mean ± SEM of three independent determinations and are a percentage of the control value, which was 13.8 ± 0.6 pmol of proline/mg of protein.

The inhibition of vesicular proline uptake by enkephalins was dose-dependent (Figure 2). Up to 60% of the proline accumulation was inhibited in the presence of the highest enkephalin concentrations used. Half-maximal inhibition occurred at approximately 1 μM [Leu]enkephalin.

Other opiates and opioid peptides were examined for their effects on vesicular proline uptake (Table I). [Met]enkephalin and [Leu]enkephalin and their des-tyrosyl derivatives had

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

Table II: Accumulation of Neuroactive Amino Acids by Plasma Membrane Vesicles Derived from Synaptosomes and Selective Inhibition by [Met]enkephalin[a]

amino acid	amino acid accumulation [dpm (mg of protein)$^{-1}$ × 10^3] control	25 μM enkephalin
aspartic acid	37.9 ± 0.6	39.3 ± 1.5
glutamic acid	104.4 ± 0.5	108.9 ± 4.8
γ-aminobutyric acid	32.7 ± 4.5	29.6 ± 2.7
glycine	15.3 ± 1.3	12.2 ± 0.6
proline	8.2 ± 0.1	3.2 ± 0.3

[a] Maximal accumulation of the amino acid was determined in the presence and absence of 25 μM [Met]enkephalin after incubations for the appropriate time periods as previously determined for vesicular uptake of aspartic acid (Marvizon et al., 1981), glutamic acid (Kanner & Sharon, 1978), γ-aminobutyric acid (Kanner, 1978), glycine (Mayor et al., 1981), and proline (Rhoads et al., 1982a). Each value is the mean ± SEM of three independent determinations.

similar inhibitory effects on proline uptake. Each of these peptides inhibited proline accumulation by 50% or more at a concentration of 15 μM. Morphine, dynorphin and [Leu^5]-β-endorphin (both of which contain the [Leu]enkephalin sequence), and β_h-endorphin (which contains the [Met]enkephalin sequence) had no significant inhibitory effect on proline uptake. The amide derivative of [Leu]enkephalin, [Leu]enkephalinamide, a potent opiate agonist, was less inhibitory compared to [Leu]enkephalin. Thus, the modification of the C-terminus of the enkephalin including extensions of the enkephalin sequence from the C-terminus (i.e., the β-endorphins and dynorphin) led to decreased effectiveness of the enkephalin in its ability to inhibit proline uptake. Modified enkephalins with greater selectivity for the δ subclass of enkephalin binding sites, DSLET (D-Ser^2-[Leu]enkephalin-Thr) and DADLE (D-Ala^2-[D-Leu^5]enkephalin), were less inhibitory than [Leu]enkephalin. Dose-dependent inhibition by DSLET was seen in the concentration range of 15–100 μM. Significant inhibition by DADLE was seen only at concentrations greater than 100 μM (data not shown).

Several amino acids with neurotransmitter properties are taken up by the synaptosomal plasma membrane vesicles under these conditions (Table II). No effect of up to 10 μM [Leu]enkephalin was observed on the uptake of glutamic acid, aspartic acid, or γ-aminobutyric acid. The vesicular uptake of glycine was inhibited by [Leu]enkephalin, although the degree of inhibition was substantially less for glycine than for proline.

[Leu]enkephalin Binding. Binding of [^{3}H]Leu-enkephalin to the plasma membrane vesicles reached a saturation level at 10–20 nM [Leu]enkephalin (Figure 3A). Under the incubation conditions employed, the binding reached equilibrium after approximately 60 min (Figure 3B). Specific binding represented 65%–75% of the total enkephalin binding measured, and the maximum specific binding represented 1–10 fmol/mg of membrane protein. Although the concentrations of [Leu]enkephalin employed to inhibit proline uptake are in excess of that needed to saturate the enkephalin binding site(s), it should be noted that the equilibrium conditions for binding of [Leu]enkephalin and for proline uptake are totally dissimilar.

The lack of naloxone antagonism and the observation that des-Tyr-enkephalins are as inhibitory as the intact pentapeptides indicated that this was a nonopioid property of the enkephalins and that this was not an effect which was mediated by opiate receptors. Since the inhibition by the des-tyrosyl peptides is, thus, one of the distinctive features of the inhibition of proline uptake by enkephalins, the effect of des-Tyr-

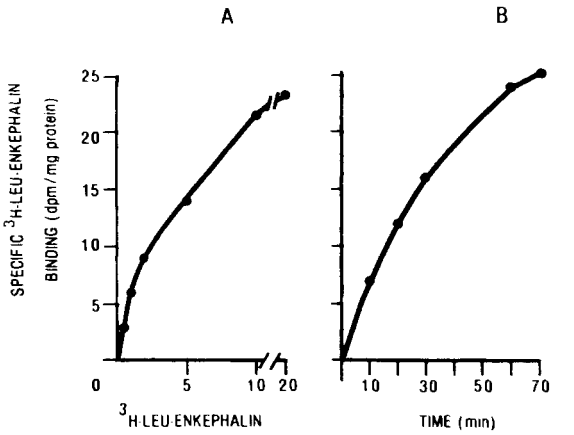

FIGURE 3: Specific [^{3}H]enkephalin binding to synaptosomal plasma membrane vesicles. (A) Vesicles were incubated for 60 min with 0.1–20 nM [^{3}H]Leu-enkephalin. (B) Vesicles were incubated with 20 nM [^{3}H]Leu-enkephalin for varying time periods. In each case, all values were corrected for nonspecific binding as determined in the presence of 20 μM unlabeled [Leu]enkephalin.

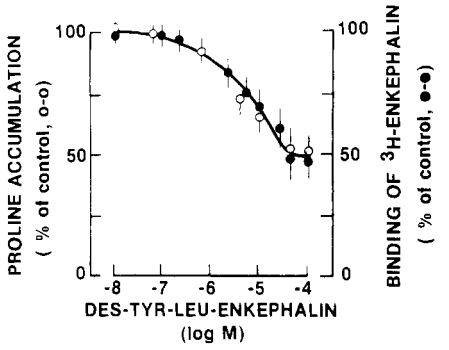

FIGURE 4: Effects of des-Tyr-[Leu]enkephalin on [^{3}H]Leu-enkephalin binding and proline transport. Specific binding of [^{3}H]Leu-enkephalin was determined after 60-min incubations with 20 nM [^{3}H]Leu-enkephalin in the presence of varying concentrations of des-Tyr-[Leu]enkephalin (●). The effects of the same concentrations of des-Tyr-[Leu]enkephalin were determined for proline uptake (O). The results are expressed as a percent of the control values obtained in the absence of des-Tyr-[Leu]enkephalin.

[Leu]enkephalin in [^{3}H]Leu-enkephalin binding to the membrane vesicle preparation was examined. Des-Tyr-[Leu]-enkephalin was found to displace up to 50% of the specific enkephalin binding (Figure 4). The concentration dependence of the displacement of [^{3}H]Leu-enkephalin binding by des-Tyr-[Leu]enkephalin is similar to that of the inhibition of vesicular proline uptake by des-Tyr-[Leu]enkephalin (Figure 4). This suggests that the des-tyrosyl peptides interact with specific enkephalin binding sites and that these binding sites may mediate the inhibition of proline uptake by enkephalins. Scatchard analyses (Scatchard, 1949) of binding data revealed one major class of sites with an apparent K_D of 2 μM. Another class of sites, with a K_D of 85 μM, was detectable, but the significance of this is as yet unknown. The K_D value of the major site correlates with the half-maximal value (1 μM) calculated for enkephalin-induced inhibition of proline uptake.

Discussion

The characteristics of the inhibition of vesicular proline uptake by enkephalins were identical qualitatively with those observed with intact synaptosomes (Rhoads et al., 1983b). The inhibition occurred with the enkephalins and des-tyrosyl-enkephalins but not with morphine or opioid peptides containing the enkephalin peptide sequences. The generally higher concentrations of enkephalins employed in these vesicle studies

compared to those of the intact synaptosomes (Rhoads et al., 1983b) may be explained in part by the mixed sidedness of the vesicle preparation. Vesicles prepared by similar methods were shown to be a mixture of inside-out and right-side-out vesicles (Gill et al., 1981). While the amino acid carriers appear to be able to move in either direction with net transport determined by the presence of specific ion gradients [see Rhoads et al. (1983a)], the inhibition of proline transport by enkephalins was vectorial in that influx but not efflux was affected (Rhoads et al., 1983b). This finding combined with a presumed localization of enkephalin binding sites on the outside of the membrane would imply that the inside-out fraction of the vesicle preparation is not susceptible to inhibition by enkephalins. This would reduce the fraction of the total proline uptake that was enkephalin-sensitive and would account for quantitative differences in the two preparations.

The demonstration that the des-tyrosyl-enkephalins were as inhibitory to proline transport as the pentapeptides was consistent with the lack of naloxone antagonism in indicating that the inhibition was due to a nonopioid property of the enkephalins. Des-tyrosyl derivatives of the β-endorphins (DeWied, 1979; DeWied et al., 1980) and dynorhins (Walker et al., 1982) have been shown to have nonopioid pharmacological activity. Such activity may represent a subfraction of the physiological effects of the parent peptides that are also shared by their des-tyrosyl derivatives and, thus, may be mediated by a subpopulation of the binding sites for the parent ligands, which also recognize their des-tyrosyl fragments. The present study demonstrates that des-Tyr-[Leu]enkephalin competes for a fraction of the specific binding of [^{3}H]Leu-enkephalin and does so in the same concentration range that proline transport is inhibited by these peptides. Since most enkephalin binding studies are carried out in Na^+-free medium due to the inhibitory effect of Na^+ [e.g., Chang et al. (1979)], we carried out binding and competition studies under conditions (150 mM NaCl) identical with those in which inhibition of proline uptake by enkephalins was observed. The lower level of binding in our studies, which was approximately 2 orders of magnitude less than that described for cortical regions by Chang et al. (1979), may be ascribed to the presence of Na^+. The possibility exists that the binding occurring under our conditions is predominantly due to a specific subclass of enkephalin binding sites rather than representing a general reduction in the enkephalin binding and that some selectivity among the putative enkephalin receptor subclasses is inherent in the binding assays.

The involvement of specific enkephalin binding sites in enkephalin-mediated inhibition of proline transport is further demonstrated by the identical nature of the inhibition curves obtained for enkephalin on [^{14}C]proline uptake and [^{3}H]-enkephalin binding. Scatchard analyses of binding data revealed a major class of enkephalin binding sites with a K_D of 2 μM. In addition to this site, a lower affinity site (K_D = 85 μM) was detectable, but its contribution to total enkephalin binding was insignificant. Moreover, the contribution by this lower affinity site on enkephalin-induced inhibition of [^{14}C]-proline uptake was not perceivable, since the maximal inhibition of [^{14}C]proline uptake, under the conditions employed in our study, was about 60% at 30 μM enkephalin. Further increases in enkephalin concentrations produce no effect on [^{14}C]proline uptake. The presence of lower affinity sites might suggest negative cooperative interactions between the two classes of sites for enkephalin in synaptosomes; this phenomenon has been reported in other cell systems (Gammeltoft & Gliemann, 1973; Gliemann et al., 1975; Sankaran et al., 1981). The observation, in the present study, of a curvilinear Scatchard plot and a Hill plot having a slope significantly less than 1.0 (0.33) suggests negative cooperative interactions between the two classes of sites. Another interpretation of the data is the possible heterogeneity of binding sites. It is difficult to express the high-affinity sites (involved in the inhibition of transport) as a function of the total number of sites due mainly to the interaction or heterogeneous nature of the binding sites. It, however, is clear from our data that 100% of the high-affinity sites have to be occupied to produce the maximal inhibition (60%) of proline uptake. Whether the site–site interaction or the heterogeneity of binding sites is the cause of the observed 60% maximal inhibition in both binding and [^{14}C]proline uptake remains to be elucidated.

Enkephalin, at concentrations that inhibited proline transport by up to 60%, had no effect on the uptake of the neurotransmitter amino acids, glutamic acid, aspartic acid, and γ-aminobutyric acid. The small but significant inhibition of glycine uptake is of interest in light of recent observations (Rhoads et al., 1984), suggesting that the proline carrier may be part of an iminoglycine transport system in brain which mediates Na^+-dependent uptake of imino acids such as proline and pipecolic acid as well as a fraction of the Na^+-dependent component of glycine uptake. In these studies, we have demonstrated the maximum inhibition of approximately 80% of the synaptosomal Na^+-dependent uptake of pipecolic acid and 40% of the Na^+-dependent glycine uptake by enkephalins and des-tyrosyl-enkephalins.

Previous studies have provided evidence that proline transport into membrane vesicles derived from synaptosomes is coupled to a concentration gradient for Na^+ (Rhoads et al., 1982a). This transport was stimulated by an electrical potential (inside negative) and was optimal in the presence of extravesicular Cl^-. Evidence was also provided that a major portion of the proline transport system is located in compartments also containing veratridine-sensitive sodium channels, which, like the veratridine-sensitive transport systems of other neuroactive amino acids (Kanner, 1980), is evidence of their association with the presynaptic neural membrane. Given the proposed role of these transport systems in the termination of neurotransmitter action, inhibition of presynaptic proline uptake by enkephalins would serve to prolong the postsynaptic action of released proline by reducing the efficiency of its reuptake into the nerve terminal. Since proline has been shown to have neuroactivity consistent with the potential for a role as an inhibitory neurotransmitter [see Giacobini (1983)], we suggest that one or more of the inhibitory effects of enkephalins may be mediated by modulation of proline transport and, hence, of proline neuroactivity. Likewise, if an iminoglycine transport system is the target of the enkephalins, then its other substrates with neuroactivity, such as pipecolic acid [reviewed by Giacobini (1983)] or glycine (Aprison & Daly, 1978), may participate in a neuromodulatory cascade involving enkephalins. Further understanding of (1) the nonopioid (i.e., nonanalgesic) effects of enkephalins and (2) the role(s) of proline and a putative iminoglycine transport system in the mammalian central nervous system will be required to elucidate the significance of these presynaptic interactions and their potential for neuromodulation.

Registry No. L-Pro, 147-85-3; Gly, 56-40-6; des-Tyr-[Leu]enkephalin, 60254-83-3; des-Tyr-[Met]enkephalin, 61370-88-5; [Met^5]-enkephalin, 58569-55-4; [Leu^5]enkephalin, 58822-25-6; Na, 7440-23-5.

REFERENCES

Aprison, M. H., & Daly, E. C. (1978) *Adv. Neurochem. 3*, 203.

Balcar, V. J., & Johnston, G. A. R. (1974) *Biochem. Pharmacol. 23*, 821.
Balcar, V. J., Johnston, G. A. R., & Stephanson, A. L. (1976) *Brain Res. 102*, 143.
Bennett, J. P., Logan, W. J., & Snyder, S. H. (1972) *Science (Washington, D.C.) 178*, 997.
Chang, K.-J., Cooper, B. R., Hazum, E., & Cuatrecasas, P. (1979) *Mol. Pharmacol. 16*, 91.
Chang, K.-J., Blanchard, S. G., & Cuatrecasas, P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 940.
DeWied, D. (1979) *Trends Neurosci. (Pers. Ed.) 2*, 79.
DeWied, D., VanRee, J. M., & Greven, H. M. (1980) *Life Sci. 26*, 1575.
Gammeltoft, S., & Gliemann, J. (1973) *Biochim. Biophys. Acta 320*, 16.
Giacobini, E. (1983) *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 3, p 583, Plenum, New York.
Gill, D. L., Grollman, E. F., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 184.
Gliemann, J., Gammeltoft, S., & Vinten, J. (1975) *J. Biol. Chem. 250*, 3368.
Iversen, L. (1971) *Br. J. Pharmacol. 41*, 571.
Kanner, B. I. (1978) *Biochemistry 17*, 1207.
Kanner, B. I. (1980) *Biochemistry 19*, 692.
Kanner, B. I., & Sharon, I. (1978) *Biochemistry 17*, 3949.
Marvizon, J. G., Mayor, F., Jr., Aragon, M. C., Giminez, C., & Valdivieso, F. (1981) *J. Neurochem. 37*, 1401.
Mayor, F., Jr., Marvizon, J. G., Aragon, M. C., Giminez, C., & Valdivieso, F. (1981) *Biochem. J. 198*, 535.
Mudge, A. W., Leeman, S. E., & Fischbach, G. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 526.
Mulder, A. H., & Synder, S. H. (1974) *Brain Res. 76*, 297.
Nicoll, R. A. (1982) *Adv. Biochem. Psychopharmacol. 33*, 337.
Peterson, N. A., & Raghupathy, E. (1972) *J. Neurochem. 19*, 1423.
Rhoads, D. E., Peterson, N. A., & Raghupathy, E. (1982a) *Biochemistry 21*, 4782.
Rhoads, D. E., Peterson, N. A., Raghupathy, E., & Verity, M. A. (1982b) *J. Neurochem. 38*, 1305.
Rhoads, D. E., Peterson, N. A., Sankaran, H., & Raghupathy, E. (1982c) *Biochem. Pharmacol. 31*, 1875.
Rhoads, D. E., Osburn, L. D., Peterson, N. A., & Raghupathy, E. (1983a) *J. Neurochem. 41*, 531.
Rhoads, D. E., Peterson, N. A., & Raghupathy, E. (1983b) *J. Biol. Chem. 258*, 12233.
Rhoads, D. E., Peterson, N. A., & Raghupathy, E. (1984) *Biochemistry 23*, 117.
Sankaran, H., Iwamoto, Y., Korc, M., Williams, J. A., & Goldfine, I. D. (1981) *Am. J. Physiol. 240*, G63.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Walker, J. M., Moises, H. C., Coy, D. H., Baldrighi, G., & Akil, H. (1982) *Science (Washington, D.C.) 218*, 1136.

Influence of Anions and pH on the Conformational Change of Horse Liver Alcohol Dehydrogenase Induced by Binding of Oxidized Nicotinamide Adenine Dinucleotide: Binding of Chloride to the Catalytic Metal Ion†

Wolfgang Maret and Michael Zeppezauer*
Fachbereich 15.2 Analytische und Biologische Chemie, Universität des Saarlandes, 6600 Saarbrücken, FRG
Received May 6, 1985

ABSTRACT: The conformational change of horse liver alcohol dehydrogenase induced by binding of NAD^+ was studied by electronic absorption spectroscopy using cobalt as a spectroscopic probe in the active site. The complex of the enzyme with NAD^+ exists in an acidic and an alkaline form. The transition between the two forms proceeds through several intermediates and is controlled by an apparent pK_a of 6.9. Only at pH values below this pK_a can a complex between enzyme, NAD^+, and Cl^- be formed. The spectral changes indicate that chloride displaces the cobalt-bound water molecule in a tetracoordinate structure. We conclude that a negative charge at the active site is necessary to stabilize the closed conformation of the enzyme in the presence of NAD^+. Spectral correlations are given which strongly support the postulation of a metal-bound alkoxide in the closed structure of the enzyme as an essential feature of the catalytic mechanism of horse liver alcohol dehydrogenase.

It is becoming apparent that the catalytic zinc ion in horse liver alcohol dehydrogenase (HLADH,[1] EC 1.1.1.1) plays a multifunctional role (Zeppezauer et al., 1984). The metal ion activates the substrate by direct coordinative bonding, thereby functioning as a Lewis Acid. In addition, it has recently been noticed that the metal ion influences the dynamics of coenzyme binding although direct bonding interactions between the coenzyme molecule and the catalytic metal ion do not exist. In spite of these insights, much more information is needed to delineate the molecular details of the catalytic cycle. In particular, a keystone to the mechanism is the number and chemical nature of intermediates formed during ternary complex interconversion. Such information has recently become

† This work was supported by the Deutsche Forschungsgemeinschaft, Bundesminister für Forschung und Technologie, NATO, FEBS, and the Universität des Saarlandes.
* Address correspondence to this author.

[1] Abbreviations: HLADH, horse liver alcohol dehydrogenase; Zn-HLADH, native zinc enzyme; Co-HLADH, active-site-specific Co^{2+}-reconstituted enzyme; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

0006-2960/86/0425-1584$01.50/0