the unfolding of $tRNA^{Phe}$ as a function of Mg^{2+} concentration (Levy, 1971; Levy and Biltonen[4]).

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Specificity and Spectral Resolution of an L-Glutamate Dehydrogenase–Monocarboxylic Amino Acid Complex†

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ABSTRACT: We recently reported differential spectroscopic evidence demonstrating the existence of an L-glutamate dehydrogenase–L-leucine complex. Extension of these spectroscopic studies shows that a variety of monocarboxylic amino acids can combine with the enzyme to form such a complex and that two groups of these complexes can be distinguished by their difference spectra. The difference spectra consist of two components: (1) a blue-shifted tryptophan perturbation spectrum which occurs in complexes of all of the amino acids and (2) a red-shifted tyrosine perturbation spectrum which appears only in complexes formed by amino acids possessing long aliphatic side chains. The ability of amino acids of one class to displace the amino acids of the other class in these complexes indicates a common binding site for all of the amino acids. The ligand requirements for complex formation and for formation of a 279-nm peak allow a simple estimation of the maximum distance between the two enzyme chromophores involved in the formation of this enzyme–ligand complex.

Recently we established the presence of a glutamate dehydrogenase–L-leucine complex with ultraviolet differential spectroscopic methods and suggested that this complex may be related to the leucine activation of the glutamate dehydrogenase reaction (Prough *et al.*, 1972). It was shown that the dissociation constant of this complex was approximately 270 μM and that the dissociation constant was independent of

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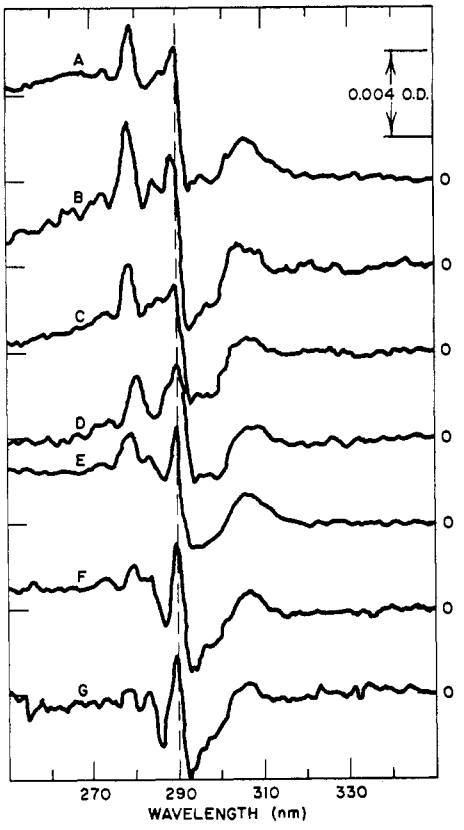

FIGURE 1: The ultraviolet difference spectrum of several L-glutamate dehydrogenase–monocarboxylic L-α-amino acid complexes. The glutamate dehydrogenase concentration was 1.00 ± 0.03 mg/ml for all of the spectra. The complexes were formed by addition of the following concentrations of appropriate amino acid: (A) 0.56 mM L-leucine, (B) 2.70 mM L-norvaline, (C) 17.60 mM L-norleucine, (D) 15.75 mM L-methionine, (E) 45.00 mM L-α-aminobutyrate, (F) 9.60 mM L-valine, and (G) 5.41 mM L-isoleucine.

enzyme concentration; *i.e.*, L-leucine did not preferentially bind to any one molecular weight species of the enzyme.

We also reported that other monocarboxylic amino acids gave similar spectra which were typical of binding difference spectra involving enzyme aromatic amino acid chromophores. This paper will describe the ligand specificity of these enzyme–monocarboxylic amino acid complexes, resolve the enzyme aromatic amino acid signals of these complexes, and discuss the specific requirements involved in the formation of the complexes.

Experimental Section

The L-glutamate dehydrogenase [L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3] and amino acids were purchased from the Sigma Chemical Co. and prepared as described previously (Prough *et al.*, 1972). The enzyme concentrations were determined from 280-nm absorbance readings obtained on a Zeiss PMQII spectrophotometer using a value of 0.97 as the extinction coefficient of a 1-mg/ml solution of glutamate dehydrogenase (Olson and Anfinsen, 1952). The enzyme had a A_{280}/A_{260} ratio of 1.95 to 1.98 and a specific activity of 4.0 ± 0.2 units per mg of protein (Prough *et al.*, 1972). A Radiometer PHM 26 pH meter was used to measure all pH values. The buffer used was 0.1 M potassium phosphate at pH 7.6.

The spectra were obtained as previously described (Prough *et al.*, 1972) on a Cary Model 14 spectrophotometer with the photomultiplier directly interfaced to a Varian 620i computer and each averaged spectrum was plotted using a Houston Model 6420 Omnigraphic recorder. The model difference spectra of the ethyl esters of *N*-acetyltryptophan (53.9 μM) and *N*-acetyltyrosine (312.5 μM) perturbed by 25% (w/v) sucrose and the model spectrum of the enzyme (0.428 mg/ml) were collected in a manner similar to that used to obtain the spectra of the enzyme–amino acid complexes. The resultant spectra which will be shown in a later section were linear combinations of model and experimental spectra obtained with the Varian computer.

TABLE I: Dissociation Constants for the Various L-Glutamate Dehydrogenase–Monocarboxylic Amino Acid Complexes.

Amino Acid	Diss Constant[a] (mM)
L-Leucine	0.30 ± 0.03
L-Norvaline	1.35 ± 0.15
L-Isoleucine	2.71 ± 0.25
L-Valine	4.81 ± 0.50
L-Methionine	7.87 ± 0.75
L-Norleucine	8.82 ± 0.75
L-α-Aminobutyrate	23.6 ± 1.50
L-Alanine	>1400[b]

[a] The dissociation constants were calculated in the same manner that the L-leucine complex dissociation constant was calculated (Prough *et al.*, 1972). [b] Spectrum may be due to some perturbation not related to binding.

Results

Specificity of the Enzyme–Monocarboxylic Amino Acid Complex. The difference spectra of several enzyme–monocarboxylic amino acid complexes at amino acid concentrations twice that of their dissociation constants are shown in Figure 1. The spectra of these complexes have the 289- to 293-nm spectral feature and the spectra of the L-methionine, L-norleucine, L-norvaline, and L-leucine complexes contain an additional 279- to 283-nm feature.[1] The 289- to 293-nm spectral feature was used to determine the dissociation constants of the various enzyme–amino acid complexes which are listed in Table I. As previously reported (Prough *et al.*, 1972), L-glutamate and L-α-aminoadipate did not give this signal nor prevent the formation of the enzyme–L-leucine complex. Several other compounds, such as D-norvaline (50 mM), α-ketobutyric acid (20 mM), α-ketoglutaric acid (10 mM), and γ-aminobutyric acid (42 mM) did not give this enzyme signal. These results suggest that only monocarboxylic L-α-amino acids can form complexes exhibiting the specific spectral features described above.

Resolution of the Spectral Features. The resolution of the binding difference spectra will be presented in terms of red- or blue-shifted difference spectra of tryptophan and tyrosine

[1] The spectrum of the enzyme–L-α-aminobutyrate complex has a small 279 nm peak but resembles the spectrum of the L-valine complex more than the other complexes which show a 279-nm peak. Throughout the rest of this paper we will consider the α-aminobutyrate complex to be similar to the L-valine complex; even though it appears to be intermediate to the two classes of this complex.

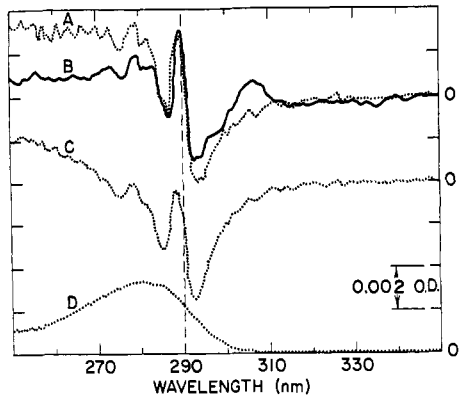

FIGURE 2: The spectral resolution of the L-isoleucine complex difference spectrum. The spectra shown correspond to the following: (A) a composite spectrum obtained by computer addition of spectra C and D, (B) a spectrum of the L-isoleucine complex difference spectrum (same as Figure 1, spectrum G), (C) a 25% sucrose perturbation spectrum of *N*-acetyltryptophan ethyl ester scaled to 27 μM (D) a spectrum of L-glutamate dehydrogenase scaled to 7.1 μg/ml.,

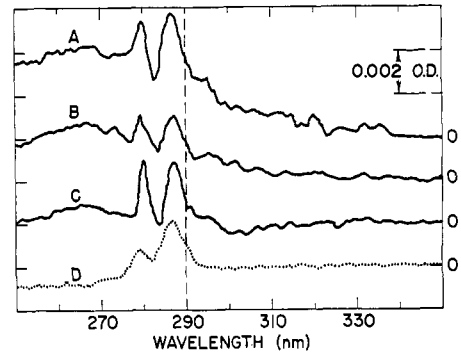

FIGURE 3: The resultant spectra of the difference between the experimental spectra of the L-isoleucine, L-valine, and L-α-aminobutyrate complex and the experimental spectrum of the L-leucine complex. The resultant spectra were obtained by the following subtractions: (A) L-leucine complex spectrum − L-isoleucine complex spectrum, (B) L-luecine complex spectrum − L-α-aminobutyrate complex spectrum, and (C) L-leucine complex spectrum − L-valine complex spectrum. Spectrum D is a 25% sucrose perturbation difference spectrum of *N*-acetyltyrosine ethyl ester (red shifted) scaled to 78 μM.

and hyperchromicities of tryptophan and tyrosine absorption. Cross and Fisher (1969) have discussed the neces of justifying the use of specific spectral components in solving binding difference spectra and have suggested certain criteria to insure that the spectral components chosen correspond to physically meaningful entities. Both red- and blue-shifted difference spectra can be produced by nonspecific perturbation of tryptophan and tyrosine analogues (Leach and Scheraga, 1960; Cross and Fisher, 1966). The spectral components used in this study will be the sucrose perturbation difference spectra of the *N*-acetyltryptophan and *N*-acetyltyrosine ethyl esters. Similar difference spectra obtained by solvent perturbation of glutamate dehydrogenase show that such chromophores exist on the surface of the enzyme and are capable of producing such shifted difference spectra. The requirements for hyperchromism as presented by Tinoco (1960) could be present in this system if the binding of the ligand alters the orientation of the enzyme chromophore. As will be seen in the following discussion, the composite spectra obtained by linear combination of these components agrees closely with the experimental spectra.

Figure 1 shows that the spectra lacking the 279-nm peak appear to be less complex and resemble the blue-shifted perturbation spectra of the ethyl ester of *N*-acetyltryptophan as seen by a direct comparison of spectra B (enzyme–L-isoleucine complex) and C (*N*-acetyltryptophan ethyl ester perturbation spectrum) in Figure 2. However, the two difference spectra differ only by a small absorption in the 280-nm region of the *N*-acetyltryptophan ethyl ester perturbation spectrum. If one adds the *N*-acetyltryptophan difference spectrum (C) to a spectrum (D) of glutamate dehydrogenase to simulate a general enzyme spectrum hyperchromicity,[2] one can obtain a composite spectrum (A) which closely resembles the spectrum (B) of the glutamate dehydrogenase–L-isoleucine complex. This correspondence of spectra A and B suggests that the enzyme chromophore perturbed by the monocarboxylic L-α-amino acid binding is a tryptophan residue.

To separate the tryptophan aromatic amino acid contribution from the difference spectra with the 279-nm peak, we subtracted the difference spectrum of the L-isoleucine, L-α-aminobutyrate, and L-valine complexes from the difference spectrum of the L-leucine complex. Figure 3 shows the resultant difference spectra and compares them to the red-shifted perturbation difference spectrum of *N*-acetyltyrosine ethyl ester. All of the spectra have the same spectral maxima and the larger 287-nm peak of each resultant spectrum has a half-bandwidth which is 87–93% of that of the *N*-acetyltyrosine perturbation spectrum. The binding of L-leucine, L-norvaline, L-methionine, and L-norleucine appears to perturb a tyrosine chromophore and a tryptophan chromophore which is perturbed by all of the amino acids which form this complex.

One additional feature of these spectra which cannot be accounted for by our resolution is the small 305-nm maximum seen in all of the spectra in Figure 1. Ananthanarayan and Bigelow (1969a,b) have studied in detail an atypical extremum around 300 nm in the difference spectra of model indole compounds and proteins containing tryptophanyl residues. They suggest that a red-shifted difference spectral feature at 300 nm may occur when the environment of a tryptophanyl chromophore changes to one with a less positive net charge. Following the argument of Ananthanarayan and Bigelow, a change in the environment of a tryptophanyl residue caused by the proximity of a carboxyl group of the α-amino acid ligand could cause a red-shifted maximum like that seen in the glutamate dehydrogenase system described in this paper.[3]

Proof for a Common Binding Site for the Enzyme–Monocarboxylic Amino Acid Complexes. At this point, it is necessary to present definitive evidence that the perturbation of the two aromatic amino acid chromophores is related to the binding of a single amino acid molecule to the enzyme. We have reported that two spectral features of the enzyme–L-leucine complex can be used to determine the dissociation constant of this complex (Prough *et al.*, 1972). The dependence of both the 289- to 293-nm and 279- to 283-nm spectral features on L-leucine concentration yield identical dissocia-

[2] Since the hyperchromic spectra of tyrosine and tryptophanyl chromophores resemble each other and the contribution of each chromophore to the apparent hyperchromicity is not known, the spectrum of the enzyme is one of the simplest models of a hyperchromicity that can be obtained for these resolutions.

[3] We thank Dr. Edmond H. Fischer for pointing out this possibility to us.

tion constants. Also, a difference spectrum resulting from the titration of a saturated enzyme–L-valine complex with L-leucine is identical to the resultant difference spectrum (Figure 3, spectrum C) obtained by subtracting the valine complex spectrum from the leucine complex spectrum. If the binding of L-valine and L-leucine perturbed tryptophan chromophores at different sites, one would expect to see the formation of a 289- to 293-nm spectral feature during the titration with L-leucine. Also, as expected from competitive binding of two ligands, the dissociation constant for the L-leucine complex determined from the 279- to 283-nm feature was increased in the presence of L-valine.

Discussion

The data presented above indicate that several monocarboxylic amino acids form an enzyme–monocarboxylic L-α-amino acid complex which can be characterized by its enzyme aromatic amino acid spectral signals. Since these ligands can displace each other, a single ligand binding site on the enzyme exists for all of these amino acids and the formation of both chromophore signals for the complexes of the longer monocarboxylic amino acids is due to the binding of single molecule.

The spectral signals can now be divided into two classes: one class which has a single 289- to 293-nm spectral feature and another class which has both 289- to 293-nm and 279- to 283-nm spectral features. The increased sensitivity afforded by linking the photomultiplier of the Cary Model 14 spectrophotometer to a Varian computer permits the resolution of the 289- to 293-nm feature into a blue-shifted tryptophan perturbation spectrum plus an enzyme hyperchromicity spectrum. The 279- to 283-nm signal can be resolved into a red-shifted tyrosine perturbation spectrum. As pointed out by other workers (Leach and Scheraga, 1960; Yanari and Bovey, 1960), several specific interactions with the chromophore can cause the observed spectra but the interaction responsible for the shifts cannot be definitively assigned.

The ability of a few of the amino acids to perturb only the tryptophan chromophore and others to perturb both the tryptophan and tyrosine chromophores can be correlated to chain length of the amino acid ligands. The longer chain ligands perturb both the tyrosine and tryptophan chromophores while the shorter amino acid ligands only perturb the tryptophan chromophores. The difference between perturbations caused by L-leucine and L-isoleucine may be due to the fact that in the crystal, L-leucine appears to exist in a more extended conformation than L-isoleucine (Subramanian, 1967; Trommel and Bijvoet, 1954). This differential signal (279 to 283 nm) which appears to depend on additional chain length allows a rough measurement of the *maximum* distance between the tryptophan and tyrosine chromophores; the distance being about 6–7 Å (the approximate length of the longest straight-chain amino acid whose complex difference spectrum does not give a 279-nm spectral peak).

Several structural requirements for the formation of this complex can be deduced from the binding constants and signal specificity. If one correlates binding ability with the length of the main aliphatic chain, the four-carbon main-chain monocarboxylic amino acids, L-leucine, L-norvaline, and L-valine, bind better than the shorter chain amino acids. Also, the dissociation constants roughly parallel the hydrophobicity of the aliphatic side-chain amino acids up to and including the four-carbon chain amino acids (Nozaki and Tanford, 1970). Steric hindrance due to the longer side chains of L-methionine and L-norleucine may decrease their binding ability and account for their dissociation constants being higher than that of L-leucine.

While the structural requirements for the amino acid ligand are somewhat speculative, they do suggest that the binding site of the enzyme may present a few rigid ligand requirements for formation of the complex. These requirements would include a hydrophobic side chain, an optimal side-chain length of four carbons, and an L-α-amino configuration.

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