The Spatial Location of Chromophoric Residues in L-Glutamate Dehydrogenase*

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ABSTRACT: Solvent perturbation difference spectroscopy was used to show the spatial location of tyrosyl, tryptophyl, and phenylalanyl residues in L-glutamate dehydrogenase. It was found that: 58% of the tyrosyl, 22% of the tryptophyl, and 75% of the phenylalanyl residues were "buried" in the anhydrous interior; there is a high concentration (39%) of tryptophyl residues in a "hole" of 2.24–4.30 A diameter; and 23% of the tyrosyl, 40% of the tryptophyl, and 9% of the phenylalanyl residues are located on the surface of the enzyme.

Previous work from this laboratory has shown that tyrosyl side-chain carboxyl hydrogen bonds hold individual δ subunits[1] (peptide chains) of L-glutamate dehydrogenase (GDH) (EC 1.4.1.3) together in larger assemblies (Fisher *et al.*, 1962a,b). More recent work has shown that tyrosyl hydrogen bonds are not broken in the α,β (dilution) split of GDH, and further that the β subunits have essentially the same chromophoric residues exposed to water as do the α units (Cross and Fisher, 1965).

While we have thus been able to study the environment of chromophoric residues and to determine the differences between α and β units as well as those between β and δ subunits of GDH by means of ultraviolet difference spectroscopy, this technique does not give direct information as to which chromophores are exposed to the solvent in the α form itself. The "solvent perturbation technique," developed by Herskovits and Laskowski (1960), does provide such information. More recently, these authors have introduced the concept that various chromophores may be located in crevices or "holes" in the protein which are accessible to small solvent molecules but not to larger ones (Herskovits and Laskowski, 1962a,b). In this way solutes of various sizes which in large concentrations do not change the conformation of the protein may be used as "molecular probes" to determine the distribution of chromophoric residues in various sized holes in the protein molecule (Laskowski, 1965). We report here the results from the application of this technique to the α form of GDH.

Experimental Procedure

Materials. L-Glutamate dehydrogenase (Lot 74B-0980, type I) was purchased from Sigma Chemical Co. *N*-Acetyl ethyl esters of L-tyrosine, L-tryptophan, and L-phenylalanine, chromatographically pure, were products of the Mann Chemical Corp. Deuterium oxide (batch XXV) was purchased from General Dynamics Corp. Sucrose, D-glucose, ethylene glycol, and mono- and dibasic potassium phosphate (all analytical reagent grade) were obtained from Mallinckrodt Chemical Works. Glycerol was supplied by Matheson Coleman and Bell.

Preparation of Solutions. L-Glutamate dehydrogenase, ammonium sulfate suspension, was dialyzed against six changes of 0.2 M potassium phosphate buffer, pH 7.6, during a 40-hr period at 4°. The resulting solution was centrifuged in a Spinco Model L ultracentrifuge for 30 min at 25,000 rpm and 4°. The supernatant was filtered through a 0.45 μ Millipore ultrafilter under nitrogen at a pressure of 2–3 psi. GDH concentrations were determined spectrophotometrically using 360, 320, and 279 mμ readings in a Zeiss PMQ II spectrophotometer. The extinction coefficient of GDH at 279 mμ was taken as $E^{1\%}_{1cm}$ 9.73 (Olson and Anfinsen, 1952). The 320 and 360 mμ readings were used for scatter correction as described previously (Fisher *et al.*, 1962b).

The *N*-acetyl ethyl esters of L-tyrosine, L-tryptophan, and L-phenylalanine were dissolved together in 0.2 M potassium phosphate buffer, pH 7.60, to give a final concentration of 3.02 mM *N*-acetyl-L-tyrosine ethyl ester, 0.465 mM *N*-acetyl-L-tryptophan ethyl ester, and 3.82 mM *N*-acetyl-L-phenylalanine ethyl ester. This stock solution was diluted 1:10 for spectrophotometric use with 0.2 M potassium phosphate buffer, pH 7.6, giving a solution containing the three chromophores in amounts equivalent to that found by amino acid analysis in 1 mg/ml of GDH.[2] Deuterium oxide solu-

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[1] The lower case Greek letters refer to a system of L-glutamate dehydrogenase (GDH) subunit nomenclature proposed by Fisher (1963).

[2] Amino acid analysis by Analytica Corp. gives the concentrations of these three amino acids in GDH as 5.49 g of tyrosine/100 g, 0.95 g of tryptophan/100 g, and 6.32 g of phenylalanine/100 g.

tions were prepared by using those amounts of mono- and dibasic potassium phosphate which gave 0.2 M solution, pH 7.60 in water. The pH of the D_2O solutions as read using glass electrodes was 7.91 ± 0.05. Stock solutions of the perturbing solutes: sucrose, D-glucose, glycerol, and ethylene glycol, were made by dissolving them in potassium phosphate buffer to give a concentration of 312.5 mg of solute/ml of 0.2 M potassium phosphate buffer solution. These stock solutions were diluted with buffer after the addition of GDH or esters to give a final perturber concentration of 250 mg/ml. GDH and model ester solutions were pipetted at constant temperature with Lang–Levy pipets which were prerinsed with the solution to be delivered and washed after delivery with diluted solution. Due to the viscous nature of some perturbants all pipets were blown out after delivery and allowed to drain completely. All solutions not containing protein were filtered through a Millipore ultrafilter (pore size 0.22 μ) to remove bacteria and to reduce scattered light.

Difference Spectrophotometric Measurements. All difference spectrophotometric measurements were made in a Cary Model 14 recording spectrophotometer equipped with a deuterium lamp. Matched pairs of square quartz cells, path length 1.000 cm, were used. The tandem cell arrangement described by Herskovits and Laskowski (1962a) was used as follows: in the sample compartment, the front cell contained the sample in buffer and a second cell contained the perturbant being used. In the reference compartment, the front cell contained the sample in the perturbing solution and the rear cell contained buffer only. This arrangement corrects for any absorbance of the perturbing solute and also corrects for scatter. All cells were inserted into thermostatted cell holders in which the temperature was maintained at 25 ± 0.05°. Difference spectrum measurements were made from an experimental base line recorded with the cells minus sample in positions as described above. When necessary, difference spectra were corrected for low differential scatter by fitting a reciprocal fourth-power overlay curve (representing scatter dependence) to the 330–450 mμ region of the difference spectrum (where absorbance is minimal). L-Tyrosine and L-tryptophan contributions to the various difference spectra were determined by measuring the height of the 285–287 mμ tyrosine peak and the 290–292 mμ tryptophan peak from the base line. Phenylalanine peaks in the 250–270 mμ range were measured not as the height from the base line but as the deviation or height from the generalized absorption in this area. This was necessary as these spectral peaks are often imposed on high slopes of differential absorption from spectral contributions in the far ultraviolet region.

Other Measurements. pH measurements were made on a Radiometer Model PHM-4 pH meter. Refractive indices were read on a Bausch and Lomb Model 3L Abbe refractometer at 25.0°. Protein concentrations as well as A_{max} for protein and ester solutions were determined using a Zeiss Model PMQ II spectrophotometer.

Errors. Dilution errors affect difference spectra as additions in the region of maximum absorption of protein or ester analogs. These were avoided by careful dilution and volumetric procedures as described above. However, some errors were still evident, and difference spectra containing these were discarded using criteria discussed in a previous paper (Fisher and Cross, 1965b). Cells were thermostatted at 25.0° for all measurements since it was found that spectral errors were significant if the temperature of the samples in the cells varied during the spectrophotometric run. Such temperature errors cause difference spectra quite similar to those produced by perturbation and therefore are difficult to detect during perturbation studies. In order to get a measurable signal it was necessary to use high concentrations of enzyme with resulting high optical densities. With this large amount of light loss, stray-light errors became a possibility. Using a deuterium lamp of high intensity and varying the slit width to check spectral reproducibility ensured that there were no contributions to the difference spectra from stray light. Actual spectrophotometric signals were sometimes

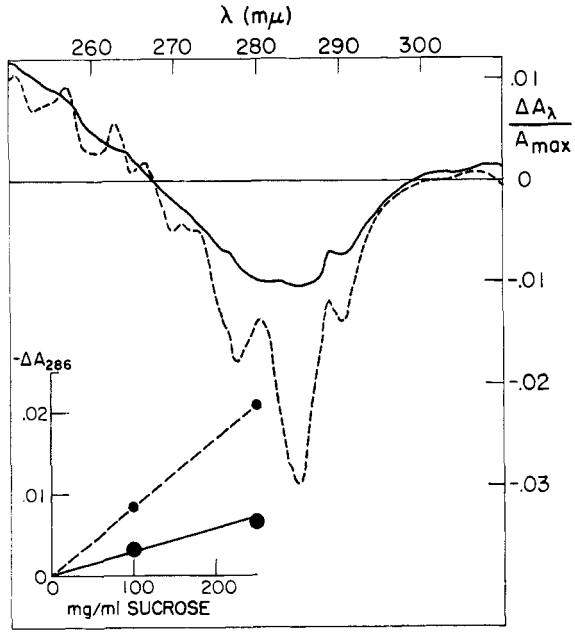

FIGURE 1: Sucrose perturbation of GDH and a model mixture. The solid line is the difference spectrum resulting from the perturbation of 1 mg of GDH/ml and the dashed line is the difference spectrum of the perturbation of a mixture of the *N*-acetyl ethyl esters of L-tyrosine, L-tryptophan, and L-phenylalanine equivalent to the occurrence of these residues in 1 mg of GDH/ml. The perturbing solution in both cases contained 250 mg/ml of sucrose. Other experimental conditions and cell arrangements are described in the Experimental Procedure. The inset shows the dependence of the differential absorption of the 285–287 mμ tyrosine peak on the perturber concentration; dashed line represents model ester dependence and solid line represents GDH dependence.

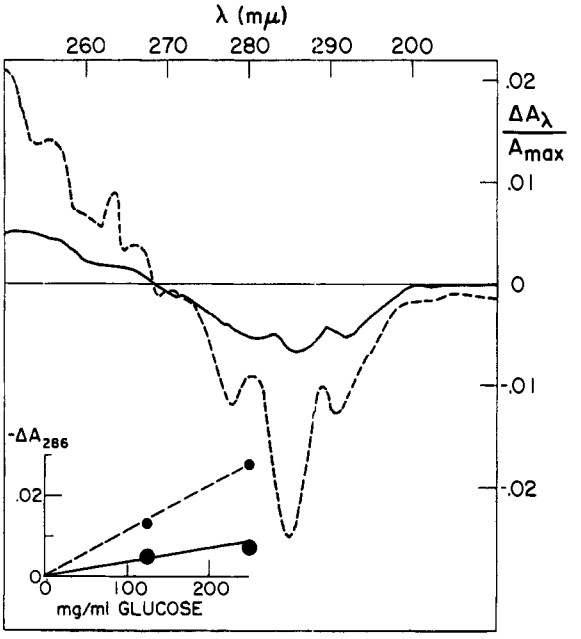

FIGURE 2: D-Glucose perturbation of GDH and a model mixture. Other experimental conditions are the same as in Figure 1 except perturber concentration was 250 mg/ml of D-glucose.

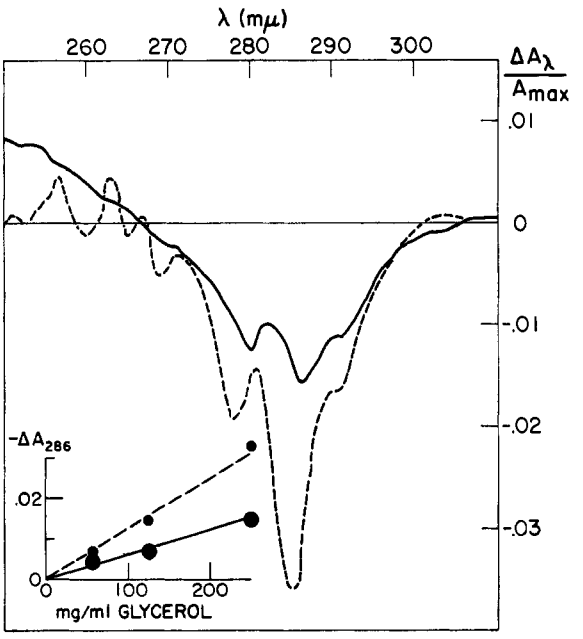

FIGURE 3: Glycerol perturbation of GDH and a model mixture. Other experimental conditions are the same as in Figure 1 except perturber concentration was 250 mg/ml of glycerol.

quite small and difficult to measure, especially the phenylalanine peaks which were of the order of 0.001 absorbancy unit on a 0–0.1 scale. To ensure accuracy, many difference spectra were measured for each set of conditions and the values obtained were averaged.

Results

The dashed lines in Figures 1 through 5 show the perturbation difference spectra of solutions containing amounts of the *N*-acetyl ethyl esters of L-tyrosine, L-tryptophan, and L-phenylalanine equivalent to the amount of these residues found in 1 mg of GDH/ml. These model perturbations are taken to be a measure of 100% exposure of the chromophores in GDH.[3] The perturbations of 1 mg of GDH/ml (solid lines) are to be compared with the model ester perturbations. Each figure represents the perturbation by a different solute having a different molecular diameter. We have previously shown (Cross and Fisher, 1965) that the sum of the perturbations of individual esters is equal to the perturbation of a mixture of these esters in which the spectral contributions of the three chromophores can be identified and measured quantitatively. Tyrosine has characteristic difference spectrum peaks in the 278–280 mμ region and in the 285–287 mμ region. Quantitative measurements for tyrosine were made from the 285–287 mμ peak since dilution error contributes to the 278–280 mμ peak. Tryptophan measurements were made from the characteristic 290–292 mμ peak, and four of the phenylalanine peaks (253, 260, 263, and 267 mμ) were used to determine the contribution of this chromophore to the difference spectra. The difference spectrum peaks obtained from GDH perturbation are displaced approximately 3 mμ to longer wavelengths than the peaks obtained from model ester perturbation. This is to be expected as the spectrum of GDH is also displaced 3 mμ to longer wavelengths than that of the model ester spectrum. All difference spectra, with the exception of those from D_2O perturbation, result from a red shift in the spectrum effected by the perturbing solute. D_2O causes a blue shift in the spectrum with a reversal in sign of the difference spectrum.[4] To compare the difference spectrum of GDH with that of the model ester mixture the actual changes in absorbancy, ΔA_λ, were not used. The relative perturbation, $\Delta A_\lambda / A_{278-280}$ (Herskovits and Laskowski, 1962a), gives

[3] The use of the *N*-acetyl ethyl ester derivatives of the three amino acid residues as a model of 100% exposure has been discussed at length by Herskovits and Laskowski (1962a). We did not use denatured GDH as a 100% exposure reference because of the possible steric hindrance to perturbing molecules. Herskovits (1964) has further shown that the chromophores in esters as compared to the chromophores in peptide chains are "very nearly fully accessible to solvent."

[4] Since both GDH and model ester perturbation by D_2O give difference spectra resulting from a blue shift in the spectrum, these $\Delta A/A$ comparisons are valid in determining the relative exposure. As can be seen in Table I, the refractive index differences for all perturbing solutions are positive with the exception of that of D_2O, demonstrating that the sense of the difference spectrum produced by solvent perturbation is relatable to the sign of the refractive index increment.

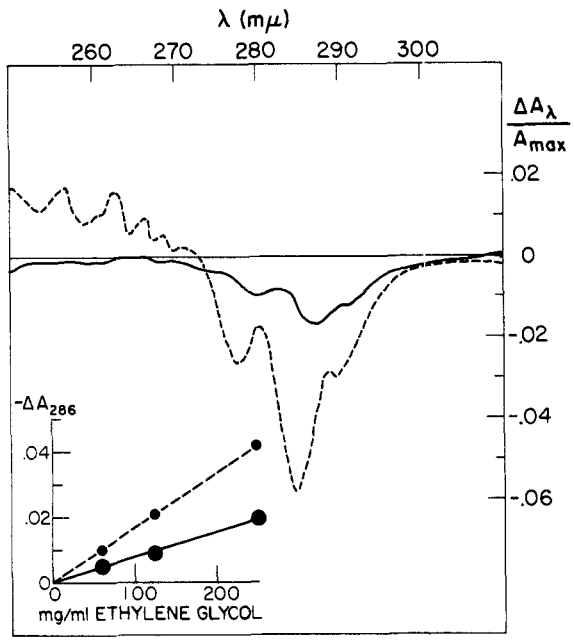

FIGURE 4: Ethylene glycol perturbation of GDH and a model mixture. Other experimental conditions are the same as in Figure 1 except perturber concentration was 250 mg/ml of ethylene glycol.

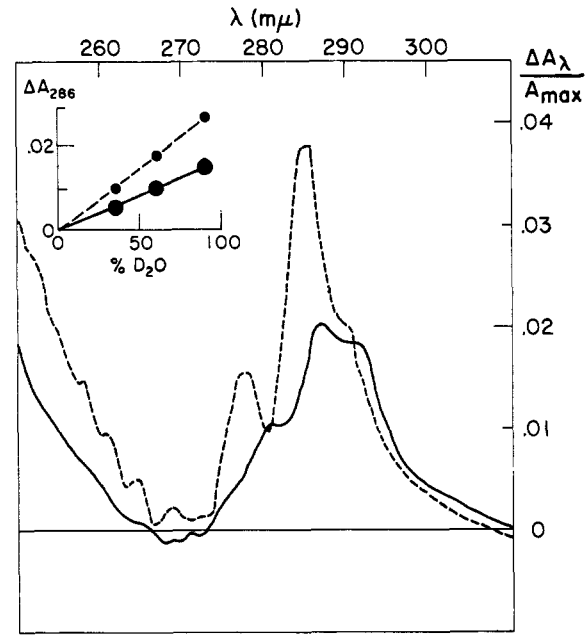

FIGURE 5: Deuterium oxide perturbation of GDH and a model mixture. Other experimental conditions are the same as in Figure 1 except perturber concentration was 89.5% deuterium oxide.

TABLE I: Fractional Exposure of Chromophores in GDH.

Perturber			Ratio of Chromophores "Seen" to Total Chromophores[b]		
Molecular Species	Diameter (A)	$\Delta n^{25°}$[a]	Tyrosine	Tryptophan	Phenylalanine
Sucrose	9.74	0.0343	0.24	0.36	0.10
Glucose	7.12	0.0345	0.27	0.44	0.16
Glycerol	5.14	0.0286	0.36	0.43	0.20
Ethylene glycol	4.30	0.0226	0.32	0.36	0.13
D_2O	2.24	−0.0043	0.44	0.78	0.24

[a] $\Delta n^{25°} = n^{25°}_{\text{perturber solution}} - n^{25°}_{\text{buffer solution}}$.
[b] $(\Delta A_\lambda/A_{max}(\text{GDH}))/(\Delta A_\lambda/A_{max}(\text{model}))$.

a more valid comparison of the chromophoric exposure to each of the perturbers. From the spectra in Figures 1–5 it can be seen that the different perturbers do "see" chromophores in GDH in varying quantities. Graphs showing the dependence of the 285–287 mμ tyrosine peak on perturber concentration are inset in Figures 1–5. Two criteria, linearity and extrapolation to the origin (Herskovits and Laskowski, 1962a) (Bigelow, 1960), ensure that there is no conformational change effected by the perturber in which the chromophores of GDH are exposed or buried. The dependence of ester perturbation on perturber concentration is included in these insets for reference. Similar plots of the tryptophan peak dependence have been obtained, and within limits of error the two above criteria were followed. As will be shown later tyrosine residues are evenly distributed throughout the protein and would be more sensitive to conformational change.

Table I lists the ratio of chromophores in GDH "seen" by the five various sized perturbers. The diameters of the perturbers and the refractive index increments which accompanied perturbation are also included in this table. The total numbers of tyrosyl, tryptophyl, and phenylalanyl residues present in GDH have been calculated from the minimum analytical molecular weight. These numbers, together with the ratios in Table I, are used to determine the actual number of chromophoric residues exposed to each perturber. The resulting values are shown in Figure 6 plotted against the diameter of the corresponding perturber.[5] It should be pointed out that if each of the molecules

[5] The diameters of the perturbing solutes were supplied to us by Dr. M. Laskowski, Jr. (personal communication), referenced as follows: sucrose, glucose, and glycerol from diffusion constants (International Critical Tables, 1929); diameter of ethylene glycol from Rossi *et al.* (1958); and diameter of water from Wang (1951). We have assumed for purposes of this paper that the diameter of deuterium oxide is the same as that of water and that the same chromophores would be perturbed by both isotopic forms of water.

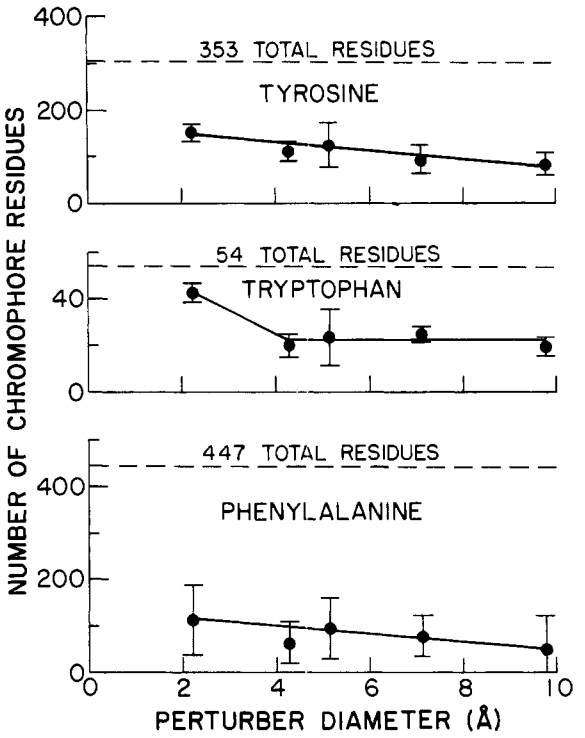

FIGURE 6: The number of tyrosine, tryptophan, and phenylalanine residues exposed in GDH *vs.* the size of perturbing molecules. The horizontal dashed line at the top of each graph shows the total number of each residue present in GDH.

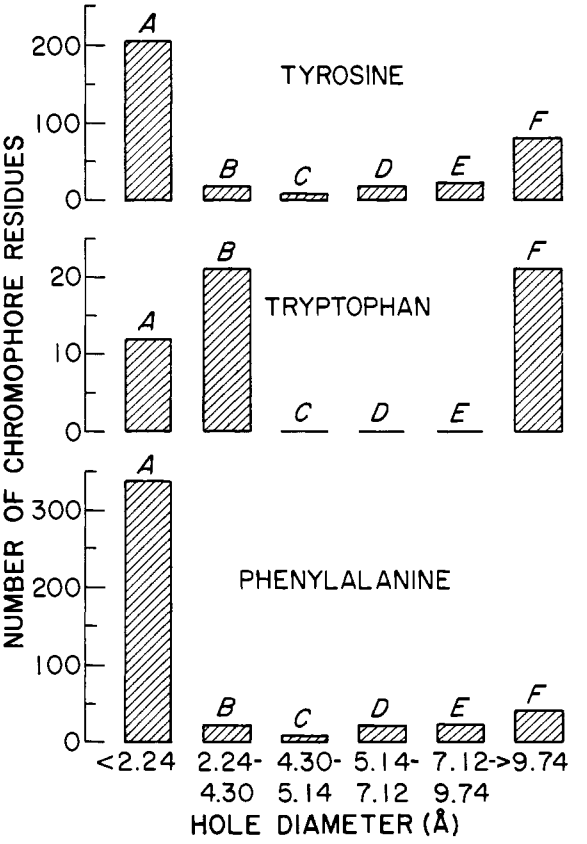

FIGURE 7: Distribution of tyrosine, tryptophan, and phenylalanine residues within spatially defined regions in glutamate dehydrogenase. Column A represents the number of residues residing within "holes" smaller than 2.24 A including residues which are completely buried. Columns B, C, and D represent the number of residues located in spatial regions as defined by perturber size. Column E shows the number of residues located in "holes" larger than 9.74 A which include the surface of GDH.

had equal "perturbing ability" it would be impossible for a small perturber to "see" less residues than a larger one. However, Herskovits and Laskowski (1962b) have stated that there are "range effects" associated with different perturbers depending on the perturber interaction with the chromophore. Thus perturber size does not strictly limit the perturbing ability. This phenomenon may account for the general inability of ethylene glycol to perturb the same number of chromophores as that of the larger glycerol molecule. Figure 6 shows that there is a generally increasing ability of the perturber to "see" the three chromophores as its size decreases. The number of chromophores obtained from the solid line, drawn as our interpretation of the data in Figure 6, is used together with perturber diameters to define six different spatial locations in GDH and to determine the distribution of tyrosyl, tryptophyl, and phenylalanyl residues within each of these locations.

In Figure 7, column A represents the number of each of the residues which are "buried" in the anhydrous interior of the protein. The majority of the phenylalanyl residues are located in this region, in fact, 338 of the total 447 residues. Of the total 353 tyrosyl residues 204 reside in this region. Only 12 of the total 54 tryptophyl residues are distributed in here. In the B, C, D, and E size holes tyrosine and phenylalanine distribution is quite even; in fact, there is a one-for-one correspondence of the appearance of tyrosine and phenylalanine in each of these holes. Tryptophan, on the other hand, exhibits a nonrandom distribution as 21 of the total 54 residues are located entirely in B-sized holes, 2.24–4.30 A. There are none of these residues present in the C, D, or E size holes. On the surface of GDH (F column) we find 81 tyrosine residues representing 23% of the total, and 41 phenylalanine residues representing only 9% of the total number. Forty per cent of the tryptophan residues are in this region.

These data have been treated as if chromophores residing in these well-defined regions are completely exposed to the perturbing medium. Herskovits and Laskowski (1962a) have pointed out that the solvent perturbation method does not distinguish between completely exposed chromophores and those with only partial exposure. Thus alternate interpretations of the data may be made and the distribution of these chromophores may be shifted to nearby "holes." For example,

tryptophan distribution could be interpreted as 42 residues with half-exposure in B size holes instead of 21 residues being completely exposed.

Discussion

Previously we have shown that there were no detectable changes in the environment of tyrosyl, tryptophyl, or phenylalanyl residues in GDH when the enzyme undergoes a threefold (α,β) dissociation upon dilution from 1.0 to 0.1 mg/ml. From the data shown here we can now state that, of the 149 tyrosyl, 42 tryptophyl, and 109 phenylalanyl residues exposed to water in α-GDH, none change their environment sufficiently to be detectable by difference spectroscopy when the protein dissociates to the three β subunits. It can be concluded that those portions of the β subunit faces which come in contact with each other to form the α-GDH monomer do not contain any of the 81 tyrosyl, 21 tryptophyl, or 41 phenylalanyl residues which are located on the surface of these subunits.

About two-thirds of all tyrosyl residues are totally buried in α- and β-GDH. About one-fourth are fully exposed on the surface.

The general absence of large proportions of phenylalanine residues on the surface and in "holes" from 2.24 to 9.74 A in diameter is the most striking feature of the distribution of these residues. The large concentration of phenylalanine residues in the anhydrous interior of the protein does suggest that they may be involved in a specific structural role in GDH, such as hydrophobic bonding.

Tryptophan residues are nonrandomly distributed in three discrete "regions" of the GDH molecule. Twelve tryptophan residues are buried in the interior of the β subunit of GDH. As predicted from the involvement of a tryptophan residue in the binding of L-glutamate (Fisher and Cross, 1965a) there are tryptophan residues located on the surface of the enzyme. Of the 21 total tryptophan residues located on the surface of α-GDH it cannot be said with certainty how many are involved in substrate binding. The presence of 21 of the total 54 tryptophan residues in a "hole" defined as having a diameter from 2.24 to 4.30 A reveals that there is indeed a discrete region in the protein with an average "hole" diameter of 3 A. This region is especially well defined since no tryptophan residues appear in neighboring regions with "hole" diameters larger than 4.30 A.

Acknowledgments

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