Fluorimetric and Spectrophotometric Studies of DPN-linked Isocitrate Dehydrogenase from Bovine Heart

PROPERTIES OF TYROSYL AND TRYPTOPHYL RESIDUES*

(Received for publication, July 15, 1974)

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SUMMARY

The emission maximum of DPN-linked isocitrate dehydrogenase in pH 7.07 buffer is shifted from 317 to 324 nm and fluorescence intensity is decreased when the excitation wavelength is varied from 270 to 290 nm; in 0.2 M KOH, where the fluorescence of tyrosyl residues is almost completely quenched, a further substantial decline in quantum yield of protein fluorescence and a red shift of the emission peak to 330 nm occur. The latter should be due mainly to tryptophyl residues. The enzyme contains 9.4 tyrosyl residues per subunit of molecular weight 42,000 determined spectrophotometrically (295 nm) at pH 13, in good agreement with a tyrosine content of 9.7 by amino acid analysis. No more than 1.1 tyrosyl residues per subunit can be detected up to pH 10.6 at 7° even upon prolonged incubation. The increase in absorption at 206 nm with increasing pH is related to loss of enzyme activity and results in a red shift of the emission maximum, and decreased fluorescence intensity. Treatment of the enzyme in a Li+-containing buffer at pH 7.5 with an excess of N-acetylglutamidazole results in (a) modification of 1.1 tyrosyl residues per subunit, (b) a 30% decrease in enzyme activity, (c) a 6-nm red shift in emission maximum, and (d) a decrease in fluorescence intensity. Manganese D-l-isocitrate (1.06 mM) prevents the acetylation of the enzyme. Deacetylation of the O-acetylated enzyme by hydroxylamine completely restores the enzyme activity and reverses the spectral changes. The acetylation studies indicate that the reactive tyrosyl residue does not participate directly in catalysis but may be involved in maintaining the proper conformation of the active enzyme center. A net of 1 of the 2 tryptophyl residues per subunit is perturbed immediately by a number of solvents. This perturbation is not affected by manganous isocitrate, whereas exposure of tyrosyl residues occurs only with time and is prevented by the substrate. The perturbation of the tryptophyl residue is accompanied by a red shift of the fluorescence emission maximum. The more exposed tryptophyl residue may contribute to the energy transfer from protein to nucleotides since the quenching of protein fluorescence upon binding of DPN+, DPNH, or ADP by enzyme results in a blue shift of the emission maximum. Manganese D-l-isocitrate (1.06 mM) quenches protein fluorescence by 16% without a shift in emission peak and does not affect the relative extent of fluorescence quenching induced by the nucleotides.

It was noted in previous studies that the interaction of DPN-linked isocitrate dehydrogenase with certain ligands which are substrates or modifiers of enzyme led to changes in protein fluorescence. Thus, on binding to the enzyme, the inhibitory nucleotides DPNH and TPNH showed substantial enhancement of nucleotide fluorescence, quenching of protein fluorescence, and energy transfer (when excited at 285 nm) from the protein to DPNH (1). The specific binding of 8-anilinonaphthalene sulfonate to the enzyme and energy transfer were demonstrated by a substantial increase in dye fluorescence intensity at an excitation wavelength of 280 nm with emission maximum shifted from 515 nm for free dye to 465 nm for bound dye and concurrent decrease of protein emission. Furthermore, the position of the emission maximum of the native enzyme at 317 nm was shifted to approximately 310 nm as the binding approached 1 molecule of 8-anilinonaphthalene sulfonate per polypeptide chain (2).

The energy transfer to nucleotides or to the dye upon binding suggested either a direct interaction with or conformational modification of the protein by the ligands involving one or more aromatic amino acid residues of the enzyme. Amino acid analyses and spectral studies have shown that isocitrate dehydrogenase contains 2 tryptophan, 9 or 10 tyrosine, and 12 phenylalanine residues per subunit of molecular weight 42,000 (3). In order to evaluate the role of aromatic amino acid residues in more detail, changes in optical properties and activity of the enzyme in response to titration with alkali, O-acetylation, and binding of nucleotides were examined. The results suggest that tryptophyl residues may contribute significantly to the fluorescence of the protein, 1 tyrosyl residue is more accessible than the others to titration with alkali and O-acetylation. This tyrosyl residue seems to contribute to the proper conformation of the active center of the enzyme. The tryptophyl residues appear to be lo-

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* This work was supported in part by Grant AM 15404 from the National Institute of Arthritis, Metabolic and Digestive Diseases, National Institutes of Health. Papers I to IV in this series by C. C. F. and G. W. E. P. on the functional groups and the active center of DPN-linked isocitrate dehydrogenase from bovine heart are Refs. 2, 3, 5, and 7.
cated in different environments of the protein since a net of 1 of the 2 residues per subunit was perturbed by solvents.

**EXPERIMENTAL PROCEDURES**

**Materials**

*N*-Acetylaminodiacette, purchased from Sigma, was purified by the method of Boyer (4) and recrystallized from benzene. Solvents used for perturbation studies were of spectral quality. Other materials were obtained from the companies cited previously (5). DPN-linked isocitrate dehydrogenase from bovine heart was purified to homogeneity by the method of Giorgio et al. (6) and assayed for activity and protein content as reported previously (5, 7).

**Methods**

**Spectrophotometric Titration**—Absorbance was measured in 1-cm light path cells over the absorbance range between 0.02 and 2.00 in a Cary 14 spectrophotometer equipped with a computer interface produced by Laviv Associates (Lakewood, N. J.). All of the data were obtained by difference spectrophotometry with an appropriate blank. Isocitrate dehydrogenase (6.3 to 23 μM) was incubated at 7° for certain periods of time in the buffer systems indicated and the spectra were scanned between 250 and 350 nm. The number of residues titrated per polypeptide chain of enzyme of molecular weight 42,000 were calculated from the difference of absorbance at 295 nm as measured against an equivalent concentration of enzyme in 0.1 M sodium-Hepes1 at pH 7.07, diluted immediately before the measurement. Slight denaturation was observed after long periods of incubation. In such samples the spectra were taken after removing the precipitates by centrifugation, and the results were corrected for dilution. The tyrosyl residues titrated were calculated from the maximum extinction coefficient of the phenoxide ion of tyrosine of 2350 (8). The buffer systems used were 0.1 M sodium-Hepes at pH 7.07, 0.2 M glycine-NaOH from pH 8.6 to pH 12, and KOH above pH 12.

**Acetylation**—Enzyme was incubated at 25°, usually with a 50- to 100-fold molar excess of N-acetylimidazole, in a buffer containing 0.05 M Tris-HCl, 0.05 M sodium borate, 10% glycerol, and 0.8 M LiSO4 at pH 7.5. The extent of O-acetylation of tyrosine residues per enzyme subunit was estimated from the decreased absorbance at 278 nm using a molar absorbance of 1180 as described by Riordan et al. (9). Labile acetyl groups were determined by the method of Balls and Wood (10) after removal of excess N-acetylimidazole by passage through a Bio-Rad P-10 column (0.5 X 10 cm) as described previously (3, 5). Deacetylation of the enzyme was carried out by incubating the modified protein in the above buffer containing 1 M hydroxylamine for 10 min at 25°.

**Fluorescence Quantum Yield**—The total emission of protein solutions was compared to that of pure tyrosine, tyrosine amide, N-acetyl tryptophan, or tryptophan excited at 280 nm. The values of Chen (11, 12) for the absolute quantum yields of tyrosine and N-acetyl tyrosine (3) were corrected for dilution. The tyrosine content was estimated within the 280 to 285-nm region after subtracting the tryptophan absorption.

**Fluorescence Quenching by Nucleotides**—The fluorescence emission spectra of 0.5 μM enzyme solutions in 0.1 M sodium-Hepes at 25° excited at 280 nm were measured after the addition of increasing concentrations of various nucleotides. The emission spectra were corrected for inner filter effect by the method of Franzen et al. (15) when the absorbance of samples exceeded 0.15.

**RESULTS**

**Spectrophotometric Titration of Phenolic Hydroxyl Groups**—The spectra of native enzyme at different pH values are shown in Fig. 1. At maximal absorbance (295 nm) of the completely dissociated phenolic groups (8) and at pH 13 (Fig. 1) it can be calculated that the enzyme contains 9.4 tyrosyl residues per polypeptide chain. This value is in good agreement with a tyrosine content of 0.7 residues per subunit obtained by amino acid analysis (3). However, under the conditions used in Fig. 1 (20 min of incubation at 0°) only about one phenolic hydroxyl was titrated below pH 11.3 (Fig. 2), and no more than 1.1 tyrosyl residues per subunit of enzyme could be detected below pH 10.6 after prolonged incubation (2 hours at 7°). Within a 5-min incubation period no group was titratable below pH 8.6 and only about 0.2 group per subunit dissociated at pH 9.0. An inflection point at pH 9.5 of this titration curve was observed. Since no more than 1.1 residues could be titrated up to pH 10.6 even after prolonged incubation (2 hours), it appears that a single relatively accessible tyrosyl residue on the protein becomes exposed under these conditions. The remaining 8 or 9 tyrosyl residues of isocitrate dehydrogenase can be titrated by increasing the pH to above 12 (Fig. 1), indicating that these groups are "buried" in the protein matrix and only become accessible to solvent after substantial disruption of protein structure by alkali. The extent of dissociation of the hydroxyl group below pH 10.6 (Fig. 2A) was related to

1 The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
loss of enzyme activity (Fig. 2). A slight deviation from linearity between loss of enzyme activity and dissociation of the hydroxyl group of the tyrosyl residue was observed (Fig. 2B).

Acetylation—The selective acetylation of tyrosyl residues of proteins by N-acetylimidazole has been studied in considerable detail (9). When DPN-linked isocitrate dehydrogenase was incubated with a 60-fold molar excess of N-acetylimidazole a partial loss of enzyme activity occurred; spectral measurements at 278 nm indicated acetylation of phenolic hydroxyl groups with time until 1.1 tyrosyl residues per subunit had been modified (Fig. 3). An identical value for the labile acetyl group content of the modified protein was obtained when assayed by the hydroxamate method of Balla and Wood (10). No further modification of tyrosyl groups or decrease below approximately 70% in residual enzyme activity could be obtained upon more prolonged incubation with the reagent or by increasing the [N-acetylimidazole]/[subunit] ratio to 180. Manganese isocitrate, the substrate of the enzyme, did not restore enzyme activity to the extent of acetylation, and calculation of the extent of modification of tyrosine residues is described under “Methods.” The theoretical direct proportionality between enzyme inactivation and dissociation of the hydroxyl group of 1 tyrosine residue per subunit is shown as a straight line. The curve represents the best visual fit of the experimental points to this relationship.

Fig. 3. Stoichiometry of inactivation by N-acetylimidazole and reactivation by hydroxylamine. Enzyme (9.2 μM) was incubated with 0.6 μM acetylimidazole and changes with time in O-acetylation of tyrosine residues and enzyme activity were recorded (O——O) until no further changes occurred (approximately 30 min). Deacetylation (▲—▲) of the derivatized enzyme, acetylated by the procedure described above, was performed by incubation with 1 mM hydroxylamine. The changes in the extent of acetylation of tyrosine residues and enzyme activity at various time points are recorded here. Complete deacetylation and restoration of enzyme activity occurred after 10 min of incubation. The conditions of incubation and measurement of acetylation, deacetylation, and calculation of the extent of modification of tyrosine residues are described under “Methods.” The arrow indicates the system in the presence of manganous isocitrate (MI) (1.0 M). Complete protection from acetylation and inactivation was observed.

Fluorescence Spectra of Isocitrate Dehydrogenase—Emission spectra of the enzyme and tryptophan were examined in 0.1 M sodium-Hepes buffer at pH 7.2 at excitation wavelengths of 270, 280, and 290 nm (Fig. 4). Varying the exciting wavelength from 270 to 290 nm led to a marked decrease in fluorescence intensity of the enzyme and an approximate 7-nm red shift of the emission maximum from 317 to 324 nm. However, an excitation wavelength-dependent shift of the emission maximum of tryptophan (347 nm) was not observed in this buffer and the fluorescence intensity of tryptophan declined only slightly when the excitation wavelength was changed from 270 to 290 nm (Fig. 4). The emission maximum of isocitrate dehydrogenase (Fig. 4) is at a considerably shorter wavelength than expected if tryptophan residues were the predominant contributors to fluorescence and suggests that tyrosyl groups contribute substantially to the over-all fluorescence. This is in accord with the red shift accompanying the change of excitation wavelengths from 270 to 290 nm.

The significant contribution of tyrosyl residues to fluorescence is also supported by the observation that O-acetylation of 1 tyrosyl residue per enzyme subunit (Fig. 3) decreased the in-
The fluorescence spectra of DPN-linked isocitrate dehydrogenase at different pH values are shown in Fig. 5. At exciting wavelengths of 270 and 280 nm there was a pronounced decrease of fluorescence intensity accompanied by a red shift with increasing pH. When excited at 270 nm, the emission peak shifted from 317 nm at pH 7.07 to 339 nm in 0.2 M KOH. The peak at 339 nm is mainly due to tryptophyl residues; however, as in the case of growth hormone (18), the position of this maximum is still at a considerably shorter wavelength than that of free tryptophan or of tryptophyl residues observed with certain other denatured proteins (19). At high pH, quenching of tryptophan fluorescence was noted and attributed to energy transfer to ionized tyrosine residues (20).

Solvent Perturbation—The effects of solvents with various dielectric constants on tryptophan and a number of indole derivatives have been studied extensively (13, 14, 21-23). In general, a decrease in the dielectric constant of solvents led to a blue shift in emission maxima and an enhancement in fluorescence intensity. However, deviations from this pattern have been reported in studies of these model compounds. A number of solvents with various polarities and viscosities were selected to study their effects on the fluorescence of isocitrate dehydrogenase. All experiments were performed at neutral pH.

In accord with previous observations (13, 21), it could be demonstrated under our experimental conditions that the fluorescence of free tryptophan was quenched by substances which would...
increase the dielectric constants of solvents such as glycerol and potassium iodide. However, the fluorescence of isocitrate dehydrogenase was unaffected by these substances, at least during the initial stage of incubation. In confirmation of earlier results (21) the fluorescence of free tryptophan was increased by D_2O, but D_2O did not enhance the fluorescence of isocitrate dehydrogenase (Table I). These results suggest that the tryptophyl residues of isocitrate dehydrogenase are located in regions of the protein not readily accessible to solvents of high polarity. Solvents with low polarities appear to change the environment of the tryptophyl group, leading to enhanced enzyme fluorescence. Ethylene glycol, propylene glycol, and dimethylsulfoxide gave the most satisfactory results of the solvents tested and were selected for the perturbation studies. The increase in fluorescence of free tryptophan or of the enzyme was linear in the solvent concentration range studied. The enzyme retained activity during the period the spectra were taken, indicating that the spectral changes were not due to protein denaturation. Approximately 1 tryptophan per enzyme subunit was perturbed by these solvents (Table I). A slight red shift was observed when dimethyl sulfoxide was used.

The results obtained by ultraviolet difference spectroscopy (Table I) indicated also that a net of 1 of the 2 tryptophyl residues per enzyme subunit could be perturbed by the addition of ethylene glycol, propylene glycol, or dimethyl sulfoxide. The extent of perturbation was nearly the same with these three solvents i.e. 0.85 to 0.95 tryptophyl residues being exposed per subunit. The optical response given by the tryptophyl residue of the enzyme could be detected immediately after diluting the enzyme into the buffer containing the perturants.

There was no significant exposure of tyrosyl residues immediately after dilution of the enzyme into buffer containing ethylene glycol, propylene glycol, or dimethyl sulfoxide; however, approximately 1 tyrosyl residue was exposed in the presence of these solvents after incubation for 30 min (Table I).

When either 8 M urea or 0.5 M sodium dodecyl sulfate was used to denature the enzyme, nearly complete exposure of the tryptophyl and tyrosyl residues of the protein was observed. Approximately 2 tryptophyl and 6 tyrosyl residues could be detected in

Table 1
Solvent perturbation studies of DPN-linked isocitrate dehydrogenase

<table>
<thead>
<tr>
<th>Perturbant (concentration)</th>
<th>Fluorescence a</th>
<th>Absorption b</th>
<th>Tyrosine absorption c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene glycol (20 to 50%)</td>
<td>0.73</td>
<td>0.95 ± 0.33</td>
<td>1.56 ± 1.20</td>
</tr>
<tr>
<td>D_2O (90% to 100%)</td>
<td>0</td>
<td>0.95 ± 0.17</td>
<td>1.45 ± 0.07</td>
</tr>
<tr>
<td>Propylene glycol (20 to 30%)</td>
<td>1.07</td>
<td>0.85 ± 0.04</td>
<td>1.15 ± 0.34</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (20 to 50%)</td>
<td>0.80</td>
<td>0.96 ± 0.04</td>
<td>1.00 ± 0.00</td>
</tr>
</tbody>
</table>

a Perturbation by fluorescence measurement was studied by the method described by Steiner et al. (13). The solutions were excited at 292 nm. The number of groups perturbed was estimated by comparison to free tryptophan.

b Measurements were done according to the method of Dyson and Noltmann (14). Difference ultraviolet spectra were taken over a range of 250 to 350 nm as described under "Methods." The number of tryptophyl residues perturbed was measured at 292 nm and 285 nm immediately after dilution. The number of tryrosyl residues perturbed was estimated at 285 nm after a 30 min incubation at 7°C by subtracting the absorbance due to tryptophan which can be measured at 292 nm as described (14, 23).

c The dl-isocitrate and Mn^{2+} were 5.3 mM and 1.3 mM, respectively. MI is the abbreviation for manganous dl-isocitrate.

0.5% sodium dodecyl sulfate, and in 8 M urea the emission of the tryrosyl and tryptophyl residues of the enzyme were clearly resolved as two peaks at 303 and 340 nm, respectively. However, a detailed study of the denatured protein has not been done.

With dimethylsulfoxide as the perturant, manganous isocitrate prevented the exposure of the tyrosyl residue of the native enzyme, but not of the tryptophyl residue (Table I).

Effect of Manganous Isocitrate and Nucleotides on Enzyme Fluorescence—It was reported previously that the protein fluorescence of DPN-linked isocitrate dehydrogenase was quenched by DPNH and TPNH, but was unaffected by up to 2.7 mM dl-isocitrate (1). The present studies confirmed these observations and showed, in addition, that 1.06 mM manganous dl-isocitrate quenched fluorescence by approximately 16%; about 50% quenching of fluorescence was obtained with 18 μM DPNH, 100 μM DPN+, or 500 μM ADP when the enzyme (6.5 μM) was titrated with increasing concentrations of these nucleotides. The reduction in fluorescence intensity by manganous isocitrate occurred without a displacement of the emission peak. However, when the total protein fluorescence was reduced by 50% upon addition of the appropriate concentrations of any one of the above nucleotides, the emission peak shifted from 317 to approximately 310 nm when excited at 280 nm. The same blue shift of the emission peak and nucleotide concentration-dependent pattern of fluorescence quenching were observed in the absence or presence of manganous isocitrate.
Approximately 1 of the 9 or 10 tyrosyl residues per subunit of DPN-linked isocitrate dehydrogenase is exposed by titration up to pH 10.6 (Fig. 1) in a time-dependent reaction (Fig. 2A) which is accompanied by inactivation of the enzyme (Fig. 2B). Since considerably more drastic conditions (above pH 12) are needed for exposure of the remaining, presumably more "buried," tyrosyl residues of the protein (Fig. 1), it is likely that the same tyrosyl residue which is titratable below pH 10.6 (Fig. 2) is accessible to acetylation by N-acetyllyimidazole. O-Acetylation of this tyrosyl residue, however, led to only partial (35%) inactivation of the enzyme (Fig. 3), suggesting that this residue is not directly involved in catalysis. Nevertheless, the protection by manganous isocitrate against O-acetylation and inactivation (Fig. 3) may indicate that this tyrosyl residue is located at or near the active center and that the O-acetylated tyrosyl residue interferes with catalytic activity due to a steric effect. Alternatively, the tyrosyl residue may be located at a site on the enzyme different from although interacting with the active center. In this case, O-acetylation of the tyrosyl residue could modify the conformation at the active center and, vice versa, binding of substrate to the enzyme could affect the accessibility of the tyrosyl residue to acetylation (Fig. 3).

The function of the tyrosyl residue of DPN-linked isocitrate dehydrogenase may be similar to that of glutamate dehydrogenase. Several investigators have reported that modification of a single tyrosyl residue per subunit of glutamate dehydrogenase did not affect enzyme activity, but decreased the response of the enzyme to the allosteric inhibitor GTP (24, 25). Preliminary studies of the kinetic behavior of the O-acetylated isocitrate dehydrogenase at pH 7.4 indicate a loss of response to ADP, the positive modifier of the heart enzyme (16, 26). Thus, while the contribution of tyrosine fluorescence to the over-all fluorescence of proteins was recognized by Weber (27), the 7-nm red shift of the protein fluorescence when excited at 280 nm (30). A similar blue shift of protein fluorescence was also noted on specific binding of the fluorescent probe 8-anilinonaphthalene sulfonate to the enzyme; this was accompanied by enhancement of dye fluorescence and energy transfer (9). The 7-nm blue shift accompanying the quenching of protein fluorescence suggests that the nucleotides or anilinonaphthalene sulfonate interset preferentially with 1 of the 2 tryptophyl residues per enzyme subunit. The tryptophan which interacts with the ligands should have a higher emission maximum than the second tryptophyl residue; however, a resolution of the spectra of these tryptophyl residues has not been attempted.

Environmental heterogeneity of tryptophyl residues has been reported in many proteins. Purkey and Galley (31) have found that DPNH preferentially quenched fluorescence and phosphorescence of 1 of the 2 tryptophyl residues of horse liver alcohol dehydrogenase. The results with isocitrate dehydrogenase are consistent with these observations.

Acknowledgment The authors wish to acknowledge the many helpful discussions with Dr. Edward Kirby.

REFERENCES

23. HERSKOVITS, T. T., AND SØRENSEN, M. (1968) *Biochemistry* 1, 2523-2532
Fluorimetric and spectrophotometric studies of DPN-linked isocitrate dehydrogenase from bovine heart. Properties of tyrosyl and tryptophyl residues.

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