Mitochondrial aspartate aminotransferase is inactivated by dicarbonyl reagents selectively modifying arginyl residues. Treatment with phenylglyoxal inactivates the enzyme with concomitant modification of 2.7 mol of arginyl residues/mol of subunit. If the reaction is performed in the presence of the transaminating substrate pair aspartate/oxalacetate, only 1.3 mol of arginyl residues/mol of subunit are labeled and the enzymatic activity remains at 75% of the original value. One particular residue, identified by peptide analysis as Arg 292, is completely protected against modification in the presence of the substrate pair, indicating a role of its guanidinium group in substrate binding. On the basis of x-ray crystallographic studies of the complex of apoenzyme with N-(5'-phosphopyridyl)-aspartate (minus pyridoxal form of the enzyme), Arg 292 has been proposed as the binding site of the distal carboxylate group (Ford, G. C., Etchele, G., and Jansonius, J. N. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2559-2563). The enzyme with blocked Arg 292 is not completely inactive, and its molecular activity toward dicarboxylic substrates is of the same order of magnitude as that of the native enzyme toward alanine, which is 10^5 times lower than that toward dicarboxylic substrates. The activity toward alanine is unchanged but the rate-enhancing effect of formate on the transamination of alanine is impaired. Formate is assumed to occupy the binding site of the distal carboxylate group (Morino, Y., Osman, A. M., and Okamoto, M. (1974) J. Biol. Chem. 249, 6684-6692). Apparently, the interaction of the distal carboxylate group of the substrate with Arg 292 underlies not only the binding specificity but also the kinetic specificity of aspartate aminotransferase for dicarboxylic substrates.

Mitochondrial aspartate aminotransferase from chicken is a dimer of identical polypeptide chains with 401 amino acid residues. Both its amino acid sequence (1) and spatial structure (2) have been determined. The two active sites are situated on opposite sides of the enzyme dimer in clefts near the subunit interface. Each active site appears to be composed of structural elements of both subunits. Crystallographic analysis using N-(5'-phosphopyridyl)-aspartate as an analog of covalent coenzyme substrate intermediates has suggested that the guanidinium groups of Arg 396 and Arg 292 might be the principal binding sites for the proximal and distal carboxylate groups of the substrate, respectively (2, 3). A third guanidinium group (Arg 266) forms an ion pair with the phosphate group of the coenzyme (2). Arg 292 is one of those active site groups which are contributed by the neighboring subunit. Previous chemical modification studies have shown that both the cytosolic (4-6) and the mitochondrial isoenzyme (4) of aspartate aminotransferase are inactivated by modification of unidentified arginyl residues with various dicarbonyl reagents.

In the present study, both the characteristics of the reaction with phenylglyoxal and the catalytic properties of the enzyme with blocked Arg 292 were found to be compatible with the notion that this residue is the binding site for the distal carboxylate group of the substrate.

EXPERIMENTAL PROCEDURES

RESULTS

Inactivation of Mitochondrial Aspartate Aminotransferase by Phenylglyoxal—Treatment of the pyridoxal form of mitochondrial aspartate aminotransferase with 1.5 mM phenylglyoxal in borate buffer results in inactivation to about 5% of the initial value within 1 h (Fig. 1). Similar inactivation is brought about by other dicarbonyl reagents (see Miniprint, Table 1S). Amino acid analysis shows that in the inactivated enzyme 2.7 ± 0.5 arginyl residues (11 analyses from 4 separate experiments) out of a total of 24/subunit (1) are lost. If the modification is performed in the presence of the transaminating substrate pair aspartate/oxalacetate only 1.3 ± 0.6 arginyl residues (9 analyses from 4 experiments) are blocked with 75% of the initial enzymic activity being retained. No amino acid residues other than arginyl appeared to be modified. Using [14C]phenylglyoxal, 2.9 mol/mol of subunit (3 separate experiments) were found to be incorporated in the absence and 1.3 mol/mol of subunit (2 experiments) in the presence of the transaminating substrate pair. Thus, the stoichiometry of the reaction is close to one phenylglyoxal moiety per modified arginyl residue. A 1:1 stoichiometry has also been found with other proteins (for a review, see Ref. 17) and may be due to formation of a complex between the phenylglyoxal arginyl cis-diol adduct and borate (18), preventing the condensation with

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U. Graf-Hausner, K. J. Wilson, and P. Christen, in preparation.

\+ Portions of this paper including "Experimental Procedures," part of "Results," Tables IS and IIS, and Figs. 2S-65 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M 1851, cite authors, and include a check for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Modified Arg 292—After modification of Arg 292, the enzyme is still capable of undergoing a very slow transamination reaction. The shifts of the absorbance maximum of the enzyme-bound coenzyme from 355 nm (pyridoxal form) to 333 nm (pyridoxamine form) on addition of the amino acid substrate cysteine sulfinate and in the reverse reaction on addition of 2-oxoglutarate were followed spectrophotometrically (Fig. 5S). The rates of these conversions are 5 orders of magnitude slower than that of the transamination reaction catalyzed by the native enzyme (Table III). The residual activity toward aspartate of a few per cent of the original value as measured in preparations of the modified enzyme (Fig. 1) is due to unlabeled enzyme. In accordance with this conclusion, the apparent Michaelis constants of a preparation of modified enzyme when measured by the coupled assay with aspartate as substrate (see "Experimental Procedures") are the same as those of native aspartate aminotransferase (34).

The protection of Arg 292 against modification by the transaminating substrate pair indicates that this residue is part of the substrate binding site. In order to examine whether Arg 292 interacts with the distal or with the proximal carboxylate group, the activity of modified enzyme toward an aromatic and an aliphatic monocarboxylic amino acid was examined (Table III). The relative decrease in activity toward phenylalanine is much smaller than that toward dicarboxylic substrates. The activity toward alanine is not affected. The decreased activity of the modified enzyme toward the dicarboxylic substrates as well as the low activity of the native enzyme toward alanine is only in part due to weak binding of the substrates, rendering it impossible to measure the activity under substrate saturation (Table III).

Formate in high concentration has been reported to enhance the alanine aminotransferase activity of aspartate aminotransferase (35). This increase is thought to be due to interaction of formate with the substrate which normally binds the distal carboxylate group, and to an ensuing conformational change favorable for catalysis (36). In native mitochondrial aspartate aminotransferase, the rate of the half-reaction with alanine is enhanced 7-fold in the presence of 1 M formate. On treatment with phenylglyoxal, the apoenzyme loses its capacity to be reactivated by pyridoxal-5'-P at the same rate and to the same extent as the holoenzyme is inactivated. However, the coenzyme absorption spectrum of the modified and reconstituted apoenzyme (see "Experimental Procedures") is indistinguishable from that of the native holoenzyme (data not shown), indicating that the binding of the coenzyme is not impaired.

Identification of the Differentially Labeled Arginyl Residue—From a sample of aspartate aminotransferase modified with [14C]phenylglyoxal in the absence of substrates a 14C-labeled tetrapeptide Ile-Arg-Pro-Met was isolated which was not found in the sample of aspartate aminotransferase modified under substrate protection (Figs. 2S-4S, Table III, and text in the Miniprint). The sequence uniquely fits the section lle295-Arg-Pro-Met479 in the primary structure of mitochondrial aspartate aminotransferase from chicken (1). It also uniquely corresponds with the same section in mitochondrial aspartate aminotransferase from pig (32) and rat (33) whose complete amino acid sequence has been reported. Thus, the residue which is modified in the absence of substrates and is completely protected in the presence of the transaminating substrate pair is Arg 292. Besides Arg 292, other arginyl residues are modified by phenylglyoxal (2.9 mol of reagent were incorporated/mol of subunit in the absence of substrate, see above). While the modification of such additional residues may account for the initial decrease in activity which is not prevented by the presence of the substrates, the modification of Arg 292 is responsible for the virtually complete inactivation which is prevented by the substrate pair (see Miniprint).

Enzymic Activity of Aspartate Aminotransferase with 2-Oxoglutarate—The values listed correspond to the ratio between the activity toward the indicated substrates and the molecular activity of native enzyme with 200 mM aspartate and 6 mM 2-oxoglutarate as substrates in 50 mM sodium borate (pH 7.5, 25 °C) which is 8900 min⁻¹. The reactions were followed by the change in absorbance at 355 nm of a solution of modified enzyme (0.04–0.07 mM) with 3% residual activity in 50 mM sodium borate, pH 7.9, 25 °C, after addition of 40 mM cysteine sulfinate, 30 mM phenylalanine, or 65 mM alanine to the pyridoxamine form, or of 40 mM 2-oxoglutarate to the pyridoxamine form (see Fig. 5S).

TABLE III
Rate of half-reaction of the modified enzyme with dicarboxylic and monocarboxylic substrates

| Substrate          | Native enzyme | Modified enzyme |
|--------------------|---------------|-----------------|
| Cysteine sulfinate | N.D.*         | 1.7·10⁻⁵        |
| 2-Oxoglutarate     | N.D.          | 1.0·10⁻⁵        |
| Phenylalanine      | 1.3·10⁻⁴      | 4.5·10⁻⁶        |
| Alamine            | 5.6·10⁻⁶      | 5.6·10⁻⁶        |
| Alamine in the presence of 1 M formate | 3.8·10⁻⁵ | 3.8·10⁻⁵ / 5.6·10⁻⁶ |

* N.D., not determined.

† Halving the concentrations of cysteine sulfinate and 2-oxoglutarate to 20 mM decreases the rate of the transamination half-reaction by only 30 to 40%. The $K_v$ values of the modified enzyme for these substrates are 35 and 11 mM, respectively.

‡ A first fast phase is followed by a second slower phase corresponding to 65% of the total reaction.
addition of alanine (65 mM) modified aspartate aminotransferase in the pyridoxal form in the presence of 1 M formate shows a biphasic decrease of the absorbance at 355 nm, reflecting its transformation to the pyridoxamine form. The rate of the initial rapid phase corresponds to an acceleration of the half-reaction as observed with the native enzyme in the presence of formate. In contrast, the rate of the second slower phase (65% of the extent of the total reaction) corresponds to that in the absence of formate (see Table III). The partial preservation of the formate effect might reflect that Arg 292 has been transformed into two different derivatives, both of them impairing the activity toward dicarboxylate substrates but only one of them abolishing the rate-enhancing effect of formate. In the enzyme which has been modified under substrate protection and thus has a free guanidinium group at Arg 292, the rate-enhancing effect of formate is fully operative. A protective effect against inactivation by phenylglyoxal similar to that of aspartate plus oxalacetate is also exerted by 1 M formate or 1 M chloride (Fig. 65). This finding supports the assumption that Arg 292 is a binding site for these anions.

**DISCUSSION**

In many proteins, the guanidinium groups of arginylic residues have been found to serve as positively charged loci for the binding of anionic ligands (37, 38). Mitochondrial aspartate aminotransferase is inactivated by three different α-dicarboxyl reagents selectively modifying arginyl residues. For this study, phenylglyoxal was preferred to 1,2-cyclohexanedione and 2,3-butanedione because it inactivated the enzyme with minimum loss of arginyl residues (Table IS) and because it can be readily synthesized from a commercially available radioactive precursor. The protection against inactivation afforded by the substrate pair aspartate/oxalacetate correlates with a decrease in the number of modified arginyl residues as indicated by both amino acid analyses (Δ = 1.4 residues/subunit) and measurements of 14C incorporation (Δ = 1.6 residues/subunit). Arg 292, the residue which is completely protected by the pair of dicarboxylate substrates, is conserved in all six known aspartate aminotransferase sequences (1, 32, 33, 39-42). The only partial protection of the pyridoxal form by single dicarboxyl keto acid substrates is probably due to their weaker binding compared to the binding of productive substrates. The identification of Arg 292 as a substrate binding site is in accord with x-ray crystallographic studies of the complex of apoenzyme with phosphopyridoxyl aspartate which may be regarded as an analog of the external aldimine coenzyme-substrate compound. These studies indicated that Arg 386 interacts with the α-carboxylate group and Arg 292 with the distal carboxylate group of the substrate moiety (2, 3). The latter residue is contributed by the neighboring subunit. Nevertheless, the two subunits of the aspartate aminotransferase dimer have proven to be functionally independent (43-48).

The experimental evidence obtained by chemical modification which supports the notion of Arg 292 being the binding site for the distal carboxylate group of the substrate may be summarized as follows. 1) The modification of Arg 292 does not affect the interaction of aspartate aminotransferase with the monocarboxylic substrate alanine. Unimpaired activity toward alanine after modification of arginylic residues has also been observed previously with the cytosolic enzyme from chicken (6). 2) The activity of the modified enzyme toward phenylalanine is decreased only 30-fold while the activity toward the dicarboxylic substrates is five orders of magnitude lower than that of the native enzyme. Kinetic studies have indicated that aromatic amino acid substrates bind to a site which does not overlap with the binding site for the distal carboxylate group (49). The binding site for the aromatic ring appears to be provided by the pocket wall opposite to Arg 292 (3) and in the modified enzyme might be partially blocked by the phenylglyoxal moiety linked to Arg 292. 3) Formate which has a rate-enhancing effect on the activity of aspartate aminotransferase toward alanine (35, 36) and aromatic substrates (49) has been suggested to occupy the binding site of the distal carboxylate group of dicarboxylic substrates. The present finding that formate as well as chloride protect the enzyme against inactivation by phenylglyoxal is consonant with this view. Modification of aspartate aminotransferase under substrate protection does not impair the increase in the activity toward alanine induced by formate. On the other hand, modification of Arg 292 in the absence of substrates abolishes the effect of formate in two-thirds of the enzyme molecules (see comments on inhomogeneity of modification under “Results”).

It should be noted that the modified aspartate aminotransferase is still capable of catalyzing transamination of the dicarboxylic substrates, though the rate is reduced by 5 orders of magnitude, compared to the native enzyme. The rate of reaction of modified aspartate aminotransferase toward dicarboxylic substrates is of the same order of magnitude as that of the native enzyme toward alanine. This coincidence is in accord with the previous notion (35) that the interaction of the distal carboxylate group of the substrate with the enzyme is important for the high rate of reaction of the enzyme with its specific, i.e. dicarboxylic, substrates. The rate-enhancing effect of formate on the reaction with alanine (35, 36) is consistent with this view and might indicate that, in addition to a positioning effect on the substrate, the charge interaction might trigger conformational changes essential for efficient catalysis. The fact that the activity toward alanine is not affected by the modification and the activity toward phenylalanine is less impaired than that toward the dicarboxylic substrates indicates that Arg 386, the putative binding site for the α-carboxylate group, is still intact. For steric reasons it might not be possible to label Arg 386 if the better accessible Arg 292 (3) is blocked with the bulky phenylglyoxal moiety. Arg 266, which according to x-ray crystallographic analysis interacts with the phosphate group of the coenzyme (2), is also not susceptible to phenylglyoxal. Phenylglyoxal-modified apo aspartate aminotransferase retained the capacity to bind pyridoxal phosphate. Similarly, no arginyl residues interacting with the coenzyme could be modified with phenylglyoxal or 1,2-cyclohexanedione in aspartate aminotransferases from other sources (4-6). Taken together, the present and previous studies on the enzyme in solution and the x-ray crystallographic data (2) indicate that Arg 292 is the binding site for the distal carboxylate group of dicarboxylic substrates. The interaction between the distal carboxylate group and Arg 292 appears to underlie not only the binding specificity but also the kinetic specificity of aspartate aminotransferase for dicarboxylic substrates.

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**REFERENCES**

1. Graf-Hausner, U. (1981) Ph.D. Thesis, University of Zürich
2. Ford, G. C., Eicbele, G., and Jansonius, J. N. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2559-2563
3. Eicbele, G. (1980) Ph.D. Thesis, University of Basel
4. Riordan, J. F., and Scandurra, R. (1975) Biochem. Biophys. Res. Commun. 66, 417-424
5. Gilbert, H. F., and O’Leary, M. H. (1975) Biochem. Biophys. Res. Commun. 67, 198-202
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6. Azaryan, A. V., Melkanik, M. L., Egorov, Ts. A., and Torchinsky, Yu. M. (1978) Biochimica et Biophysica Acta 43, 696–698
7. Gehring, H., Christen, P., Eichele, G., Glor, M., Jansonius, J. N., Reimer, A.-S., Smit, J. D. G., and Thaller, C. (1977) J. Mol. Biol. 115, 97–101
8. Birchmeier, W., Wilson, K. J., and Christen, P. (1973) J. Biol. Chem. 248, 1751–1759
9. Jenkins, W. T., and D’Ari, L. (1966) J. Biol. Chem. 241, 2845–2854
10. Riley, H. A., and Gray, A. R. (1944) in Organic Synthesis (Blatt, A. H., ed) Vol. 2, pp. 509–511, Wiley, New York
11. Chang, J. Y., Brauer, D., and Wittmann-Liebold, B. (1978) FEBS Lett. 93, 203–214
12. Chang, J. Y., Creaser, E. H., and Bentley, K. W. (1976) Biochem. J. 153, 607–611
13. Laskey, R. A., and Mills, A. D. (1975) Eur. J. Biochem. 58, 335–341
14. Shrawder, E. J., and Martinez-Carrion, M. (1973) J. Biol. Chem. 248, 2140–2146
15. Riordan, J. F. (1973) Biochemistry 12, 3915–3923
16. Patthy, L., and Smith, E. L. (1975) J. Biol. Chem. 250, 557–564
17. Mornet, D., Pantel, P., Audenard, E., and Kassab, R. (1979) Eur. J. Biochem. 100, 421–451
18. Werber, M. M., Moldovan, M., and Sokolovsky, M. (1975) Eur. J. Biochem. 53, 207–216
19. Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179
20. Schloss, J. V., Norton, I. L., Stringer, C. D., and Hartman, F. C. (1978) Biochemistry 17, 5626–5631
21. Phillips, M., Pho, D. B., and Pradel, L.-A. (1979) Biochim. Biophys. Acta 566, 296–304
22. Gehring, H., Rando, R. R., and Christen, P. (1977) Biochemistry 16, 4832–4836
23. Takahashi, K. (1977) J. Biochem. 81, 395–402
24. Boeker, E. A., and Snell, E. E. (1972) Enzymes, 3rd Ed. 6, 217–253
25. Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958) J. Biol. Chem. 232, 549–558
26. Hirs, C. H. W. (1967) Methods Enzymol. 11, 199–203
27. Steers, E., Jr., Craven, G. R., Anfinsen, C. B., and Bethune, J. L. (1965) J. Biol. Chem. 240, 2478–2484
28. Berghäuser, J., and Schirmer, R. H. (1978) Biochim. Biophys. Acta 537, 428–435
29. Weng, L., Heinrikson, R. L., and Westley, J. (1978) J. Biol. Chem. 253, 8109–8119
30. Malinowski, D. P., and Fridovich, I. (1979) Biochemistry 18, 5909–5917
31. Bond, M. W., Chiu, N. Y., and Cooperman, B. S. (1980) Biochemistry 19, 94–102
32. Kagamiyama, H., Sakakibara, R., Tanase, S., Morino, Y., and Wada, H. (1980) J. Biol. Chem. 255, 6153–6159
33. Huynh, K. Q., Sakakibara, R., Watanabe, T., and Wada, H. (1980) Biochem. Biophys. Res. Commun. 97, 474–479
34. Sandmeier, E., and Christen, P. (1980) J. Biol. Chem. 255, 10284–10289
35. Saier, M. H., Jr., and Jenkins, W. T. (1967) J. Biol. Chem. 242, 101–108
36. Morino, Y., Osan, A. M., and Okamoto, M. (1974) J. Biol. Chem. 249, 6684–6692
37. Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977) Science 195, 884–886
38. Riordan, J. F. (1979) Mol. Cell. Biochem. 26, 71–92
39. Ovchininnikov, Yu. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Yu., Lipkin, V. M., Abdulasev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L., and Nosikov, V. V. (1973) FEBS Lett. 29, 31–34
40. Doonan, S., Doonan, H. J., Hanford, R., Vernon, C. A., Walker, J. M., Airoldi, da S., L. P., Boss, F., Barra, D., Carloni, M., Farrella, P., and Riva, F. (1976) Biochim. J. 149, 497–506
41. Shlyapnikov, S. V., Myasnikov, A. N., Severin, E. S., Myagkov, M. A., Torchinsky, Yu. M., and Braunstein, A. E. (1979) FEBS Lett. 106, 385–388
42. Barra, D., Martini, F., Montarani, G., Doonan, S., and Bossa, F. (1979) FEBS Lett. 108, 103–106
43. Schlegel, H., and Christen, P. (1974) Biochim. Biophys. Acta 352, 117–123
44. Schlegel, H., and Christen, P. (1978) Biochim. Biophys. Acta 532, 6–16
45. Lee, Y.-H., and Churchich, J. E. (1975) J. Biol. Chem. 250, 5604–5608
46. Boettcher, B., and Martinez-Carrion, M. (1975) Biochemistry 14, 4528–4531
47. Boettcher, B., and Martinez-Carrion, M. (1976) Biochemistry 15, 5667–5684
48. Schlegel, H., Zaozalek, P. E., and Christen, P. (1977) J. Biol. Chem. 252, 5835–5838
49. Movrides, C., and Christen, P. (1978) Biochim. Biophys. Acta 383, 769–773
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The two enzyme samples were labeled with $^{14}C$-pyridoxal or $^{35}S$-pyridoxamine in the presence and in the absence of $10^{-4}$ M pyridoxal phosphate, as described in the text. The rates of radioactive release after the indicated fragmentation and purification steps are shown.

| Fragmentation and Purification | % of Initial | % of Initial |
|-------------------------------|-------------|-------------|
| Carboxypeptidase, collagenase, | 90          | 48          |
| Proteinase K (Fig. 3)          | 12          | 5           |
| Pyridoxal 1500                  |             |             |
| Pyridoxine 1500                  |             |             |
| Pyridoxamine 1500               |             |             |

Fig. 1 - Effect of time on the rate of modification of aspartate aminotransferase (synthetic form) with chromophores. The enzyme solution (0.2 M sodium phosphate, pH 7.5) was exposed to 0.04 mM pyridoxal phosphate in the absence of arginine (A), in the presence of 7.5 mM arginine (B), and in the presence of 0.4 mM arginine (C). The tryptophan content of the enzyme was determined by a modification of the method of Ellman et al. (1960). The tryptophan content of the enzyme was very similar to that of the derivative of free arginine.

Fig. 2 - Wavelength of absorption of pyridoxal (A) and pyridoxamine (B) at various times. The absorption was measured at 330 nm and 360 nm. The absorption at 330 nm was measured at 25% of the total absorbance at 360 nm.
Chemical modification of a functional arginyl residue (Arg 292) of mitochondrial aspartate aminotransferase. Identification as the binding site for the distal carboxylate group of the substrate.
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