0273-2289/96/6101-02—0047$07.50

# D-Glyceraldehyde-3-Phosphate Dehydrogenase

## Properties of the Enzyme Modified at Arginine Residues

NATALIA K. NAGRADOVA,* ELENA V. SCHMALHAUSEN, PAVEL A. LEVASHOV, REGINA A. ASRYANTS, AND VLADIMIR I. MURONETZ

*A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia*

## ABSTRACT

Examination of the properties of *Escherichia coli* and rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenases (GPDHs) modified by 2,3-butanedione has shown that both tetrameric enzymes are stabilized, on selective modification of arginine residues (probably Arg 231), in an asymmetric state with only two active centers capable of performing the dehydrogenase reaction. The functionally incompetent active centers can be alkylated by iodoacetate or iodoacetamide in the case of *E. coli* enzyme, but are inaccessible for these reagents in the case of rabbit muscle D-GPDH. These results are consistent with the idea that the two homologous enzymes share common principles of the protein design, but differ somewhat in their active centers geometries. Modification of the arginine produces marked changes in the shape of the charge transfer complex spectrum in the region of 300–370 nm, suggestive of the alterations in the microenvironment of the nicotinamide ring of $NAD^+$, although the coenzyme binding characteristics remain largely unaltered. On arginine modification, the enzyme becomes insensitive to the effect of AMP on the kinetic parameters of *p*-nitrophenyl acetate hydrolysis reaction.

**Index Entries:** D-glyceraldehyde-3-phosphate dehydrogenase; arginine residue; active center; chemical modification.

*Author to whom all correspondence and reprint requests should be addressed. E-mail: lenka@fac.genebee.msu.su.

## INTRODUCTION

D-glyceraldehyde-3-phosphate dehydrogenase (GPDH) catalyzes the reversible oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate by the mechanism involving two steps: dehydrogenation of the substrate and phosphorolysis of the acyl intermediate, 3-phosphoglyceroyl-enzyme. The enzyme is a tetramer composed of identical subunits, which exhibit half of the site's reactivity and cooperativity in $NAD^+$ binding (*1*). Despite the extensive studies performed on this enzyme, the structural basis of its functioning is not completely understood. Crystallographic data are only available for the binary enzyme–$NAD^+$ complexes (*2,3*), and conformations of different functionally important states remain to be determined. To elucidate the contribution of individual amino acid residues to ligand binding and catalysis, the site-directed mutagenesis has been successfully employed (*4–6*). The approach used in our laboratory relies on selective chemical modification of a functionally important residue that makes it possible to change the properties of the residue without replacing it. Characteristics of the modified enzyme may provide information on the microenvironment of the site of modification and on the perturbations in the system of intramolecular interactions brought about by the modifier. In our work, GPDH modified at arginine residues was studied. Only one arginine/subunit was accessible to modification by 2,3-butanedione in the rabbit muscle enzyme (*7*); this is also true for GPDH from *B. stearothermophilus* (*6*). The arginine residue was identified as Arg 231 (*6,8*), located in a flexible, $\beta_3-\beta_2-$S-loop region of the GPDH catalytic domain, and being a component of the active center.

## MATERIALS AND METHODS

Purification of rabbit muscle GPDH and preparation of the apoenzyme were performed as previously described (*7*). *E. coli* GPDH was prepared by a procedure to be described elsewhere. Modification of arginine residues was carried out in the presence of 70 or 30 m*M* of 2,3-butanedione with the *E. coli* and rabbit muscle enzymes, respectively. At fixed time intervals, aliquots were taken to determine activity in the standard assay mixture. Owing to the inhibitory effect of borate on GPDH activity, this reagent was not used to stabilize the product of modification, and 2,3-butanedione-induced inactivation was reversible. For this reason, most of the experiments with the modified enzyme were carried out in the presence of 2,3-butanedione.

Absorbance of the enzyme–$NAD^+$ complex at 370 nm was measured in 50 m*M* HEPES, pH 8.1, 1 m*M* EDTA, 0.3 m*M* dithiothrietol, 8.25 $\mu M$ GPDH, and 1 m*M* $NAD^+$ at 15°C with the *E. coli* GPDH, and in 50 m*M* veronal, pH 8.0, 1 m*M* EDTA, 7 $\mu M$ GPDH, and 0.5 $NAD^+$ at 22°C with

rabbit muscle enzyme. Reference cells contained no enzyme. Absorption spectra of the 2,3-butanedione-modified rabbit muscle holo-enzyme were read using an enzyme sample modified in 50 m*M* HEPES with subsequent stabilization of the product of arginine modification and removal of an excess of 2,3-butanedione. The spectra of the native and modified holo-enzymes were read at 10°C in 30 m*M* MES, pH 6.5, 5 m*M* EDTA, 0.5 m*M* dithiothrietol, and 0.5 m*M* $NAD^+$.

Hydrolysis of *p*-nitrophenyl acetate was followed spectrophotometrically according to previously described procedure (*9*). The reaction was carried out in 30 m*M* veronal, pH 8.0, in the presence of 1 m*M* EDTA, 1 m*M* *p*-nitrophenyl acetate, and 3 $\mu M$ rabbit muscle enzyme. The sample containing the modified enzyme was supplemented with 30 m*M* 2,3-butanedione. D-glyceraldehyde-3-phosphate and 1,3-diphosphoglycerate were synthesized as described by Szewczuk et al. (*10*) and by Furfine and Velick (*11*), respectively.

Alkylation of GPDH by $^{14}$C-iodiacetate and by $^{14}$C-iodoacetamide (Amersham, 52–60 mCi/mmol) was performed as follows. The rabbit muscle enzyme (9 $\mu M$) was incubated with 2.8 m*M* $^{14}$C-iodoacetate either in the absence or in the presence of 6*M* urea. The samples containing the 2,3-butanedione-modified enzyme were supplemented with 30 m*M* 2,3-butanedione. Following 45 min of incubation at 20°C, 2-mercaptoethanol was added (final concentration, 30 m*M*), and after 1 min the protein was precipitated by trichloroacetic acid (final concentration 10%). The precipitate was washed with 10%, then twice with 5% trichloroacetic acid, and finally with ethanol. After drying, the precipitate was solubilized in 5*N* KOH for 3 h at 56°C, and radioactivity of the neutralized solution was measured with a scintillator. In the case of *E. coli* GPDH, the procedure was the same, but all samples contained 0.5 m*M* dithiothreitol, $^{14}$C-iodoacetamide was used, and 2,3-butanedione was present at 70-m*M* concentration. Spectral measurements were done on a Cary 219 spectrophotometer.

## RESULTS AND DISCUSSION

### Pairwise Nonequivalence of Active Centers is a Common Property of Rabbit Muscle and *E. coli* GPDH Modified by 2,3-Butanedione

Previously we have shown that on modification of a single arginine per subunit of the rabbit muscle GPDH, tetrameric enzyme is stabilized in an asymmetrical state with only two active centers capable of functioning. To test the suggestion that the capability of being locked in a state with pairwise active sites nonequivalence reflects some general principles of the protein design, analogous experiments were performed with the enzyme isolated from another source, namely *E. coli*. The studies were intended to determine the number of catalytically competent active centers

Table 1
Determination of the Number
of Functioning Active Centers of the Native
and 2,3-Butanedione-Modified GPDH

| Enzyme preparation | Enzyme source | |
|---|---|---|
| | Rabbit muscle[a] | *E. coli* |
| Native | 3.9 | 4.0 |
| Modified | 2.0 | 2.2 |

[a]Data from (7).

Reaction mixture contained: 30 m*M* veronal buffer, pH 8.3 (22°C), 5 m*M* EDTA, 1 m*M* dithiothreitol, 6.2 m*M* $NAD^+$, 0.5 m*M* D-glyceraldehyde-3-phosphate, 3.0 $\mu M$ enzyme. The sample with the modified enzyme also contained 70 m*M* 2,3-butanedione.

in the enzyme preparation subjected to total modification of arginine residues that are accessible to 2,3-butanedione. As shown in Table 1, the results are similar to those obtained with the rabbit muscle enzyme.

Along with this similarity, the enzymes display specific differences. First, modification of the arginine residues affects their catalytic activities differently (95–98% activity loss of the rabbit muscle GPDH and 78–82% of the *E. coli* enzyme). Second, the complete effect is achieved in presence of 30 and 70 m*M* 2,3-butanedione in the case of rabbit muscle and *E. coli* enzymes, respectively. The first difference probably reflects a different contribution of the modifiable arginines to catalysis in the two cases. The second one might be accounted for by inequalities of equilibrium constants in the reaction of arginine residues with 2,3-butanedione.

Figure 1 shows the time-course of the reaction of the Cys 149 residues located at the active centers of the tetrameric *E. coli* and rabbit muscle enzymes, with iodoacetamide. The reaction was followed by the disappearance of specific absorption band owing to the formation of GPDH–$NAD^+$ complex. As follows from the data in Fig. 1A, both the native and modified *E. coli* enzymes exhibited similar molar absorbancies at 370 nm (the Racker band region (*12*)) and similar time-courses of the absorbance changes caused by addition of the alkylating reagent. According to these data, all four active centers of the 2,3-butanedione-modified *E. coli* enzyme bound $NAD^+$ and were accessible to iodoacetamide. The latter conclusion is supported by the experiments on the incorporation of $^{14}C$-iodoacetamide into the protein (Table 2).

The results obtained with rabbit muscle GPDH (Fig. 1B, Table 2) revealed a characteristic difference between the two homologous enzymes. In this case, only half-active centers of the modified tetramer could react with the alkylating reagent with concomitant disappearance of the charge transfer complex. This suggests that specific differences may exist in the spatial organization of the active centers of the *E. coli* and rabbit

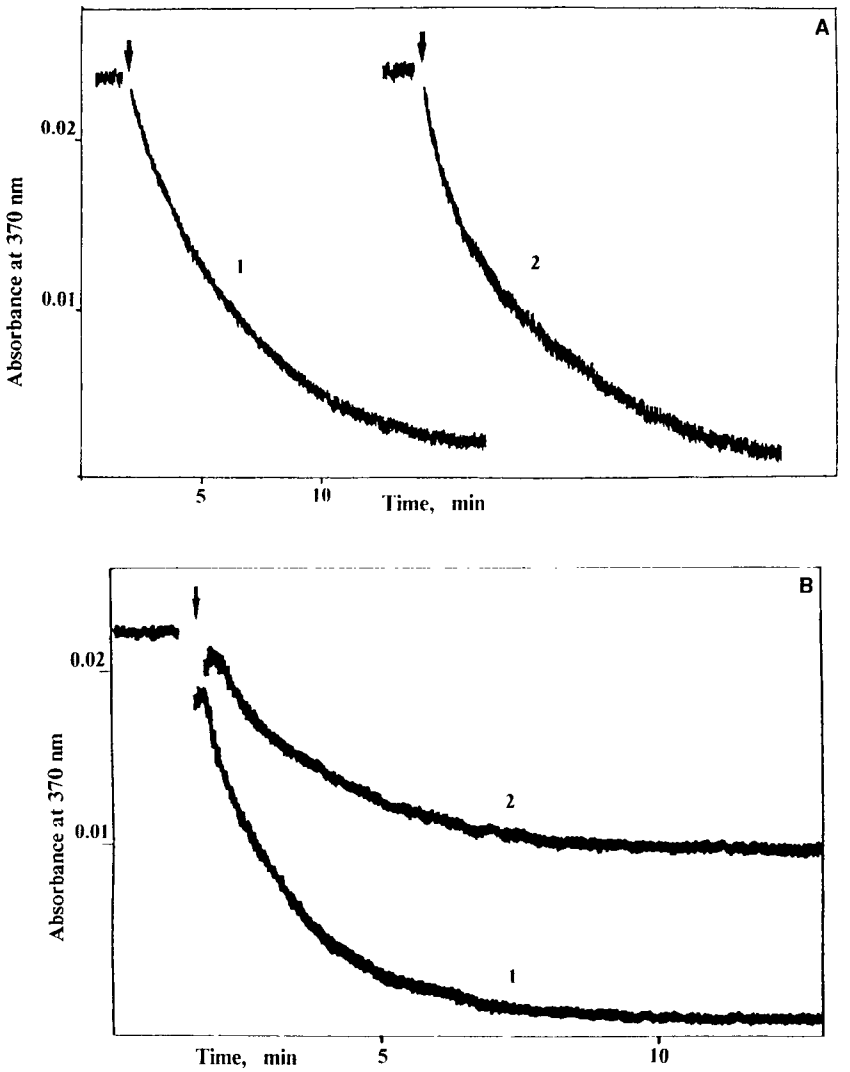


Fig. 1. Effect of iodoacetamide on the absorbance of the enzyme-$NAD^+$ complex formed by *E. coli* (**A**) and rabbit muscle (**B**) GPDH. (A) 1, Native enzyme, 2, 2,3-butanedione-modified enzyme. Sample 2 was supplemented with 70 m*M* 2,3-butanedione. At the moment indicated by the arrow, iodoacetamide was added to 0.8-m*M* final concentration. (B) 1, Native enzyme, 2, 2,3-butanedione-modified enzyme. Sample 2 was supplemented with 30 m*M* 2,3-butanedione. At the moment indicated by the arrow, iodoacetamide was added to 0.2-m*M* final concentration.

muscle GPDHs. Both enzymes are stabilized, on arginine modification, in an asymmetric state wherein two active centers may be inaccessible to substrate or incapable of properly binding it, but steric constraints may be more stringent in the case of the rabbit muscle enzyme. Taken together, the results of these comparative studies substantiate the conclusion that

Table 2
Incorporation of $^{14}$C-alkyl Groups in the Native and 2,3-Butanedione-Modified GPDHs from Rabbit Muscle and *E. coli*

| | Enzyme preparation | Rabbit muscle GPDH | | *E. coli* GPDH | |
|---|---|---|---|---|---|
| | | Incorporation of $^{14}$C-carboxymethyl groups mol/mol enzyme | | | |
| | | 1 | 2 | 1 | 2 |
| A | Native | 3.8 ± 0.2 | 0.1 ± 0.1 | 3.9 ± 0.4 | 0.2 ± 0.1 |
| | Modified | 2.2 ± 0.1 | 0.1 ± 0.1 | 3.65 ± 0.4 | 0.2 ± 0.1 |
| B | Native | 16.4 ± 0.4 | 11.6 ± 0.4 | 10.8 ± 0.8 | — |
| | Modified | 15.6 ± 0.4 | 10.9 ± 0.3 | — | — |

A and B, the treatment was performed in the absence or in presence of 6*M* urea, respectively. 1, the procedure was as described in Materials and Methods. 2, A 25-min incubation with no radiolabeled alkylating reagent preceded the treatment.

diminishing the number of functioning active centers in the modified enzyme is not a result of their deterioration or incapability of binding $NAD^+$. The data are rather suggestive of the transition of the tetramer into a particular conformational state.

The effect of chemical modification of Arg 231 on the conformational state of the tetramer probably results from specific changes in a system of intramolecular interactions. The changes may be caused by the introduction of a substituent at the guanidine group of the arginine altering electrostatic effect of this residue, by elimination of certain hydrogen bonds possibly formed by the arginine or by new interactions that may be formed by the adduct of the arginine with 2,3-butanedione. At the present stage of our knowledge, neither of these possibilities can be excluded. An important point is however that in any case an inherent asymmetry in the microenvironment of the arginine residue should be supposed. According to the results of X-ray studies (*2*), Arg 231 is located in the $\beta_3$ strand and hydrogen bonded to Ser 206 within the $\beta_2$ strand of the catalytic domain of the enzyme; a flexible loop connects the $\beta_2$ strand with the strand $\beta_1$, which comprises the essential His 176 residue. This flexible region of the protein molecule is supposed to play an important role in subunit communication (*13*). We may speculate that conformational asymmetry of the tetramer depends on specific interactions formed in this region, and differences in the accessibility of Cys 149 to iodoacetamide exhibited by the *E. coli* and rabbit muscle enzymes partially originate from structural differences at this region of the protein.

## Specificity of the Effect Caused by 2,3-Butanedione Modification on the Functional Properties of GPDH

In our previous study, it was established that modification of the rabbit muscle GPDH with 2,3-butanedione does not affect the rate of

Table 3
Rate Constants of the Acyl-enzyme Formation ($k_1$) and Acyl-Enzyme Hydrolysis ($k_2$) Steps of the *p*-Nitrophenyl Acetate Hydrolysis Reaction Catalyzed by Rabbit Muscle GPDH[a]

| | Enzyme preparation | $k_1$, $min^{-1}$ | $k_2$, $min^{-1}$ |
|---|---|---|---|
| 1 | Native | 4.17 | 0.067 |
| | Modified | 2.08 | 0.069 |
| 2 | Native | 5.71 | 0.043 |
| | Modified | 1.82 | 0.063 |
| 3 | Native | 3.46 | 0.069 |
| | Modified | 1.99 | 0.084 |

[a]Conditions: pH 7.4, 40 m*M* MOPS, 1 m*M* *p*-nitrophenyl acetate, 5 m*M* EDTA, 20°C.

*p*-nitrophenyl acetate hydrolysis catalyzed by the enzyme (*9*). Table 3 presents the rate constants of the two steps of the reaction. It is seen that the rate constant of the slow rate-limiting acyl-enzyme hydrolysis step is practically unchanged on modification. This is in contrast with the effect of arginine modification on the reaction of D-glyceraldehyde-3-phosphate oxidation, which is inhibited by 95–98%. Since *p*-nitrophenyl acetate hydrolysis is catalyzed by an apo-form of the enzyme, whereas the dehydrogenase reaction is performed by the enzyme–$NAD^+$ complex, the difference in the two cases may be explained by different positions of this residue in the apo and holo conformations of the active center. Indirect evidence for the participation of Arg 231 in the coenzyme-induced conformational changes is the rate-accelerating effect of $NAD^+$ on the enzyme inactivation by 2,3-butanedione (*14*). As shown below, the integrity of Arg 231 may also be necessary to allow conformational changes induced by other nucleotide ligands, in particular, adenine nucleotides, to occur.

We have studied the effect of AMP on hydrolysis of *p*-nitrophenyl acetate. As shown in Fig. 2, a marked rate acceleration of the enzyme acylation step occurred in the presence of AMP, when the native enzyme was used. This phenomenon initially described by Francis et al. (*15*), can be attributed to an allosteric effect of AMP bound at specific sites on the enzyme molecule changing the reactivity of Cys 149. On arginine modification, the effect disappeared (Fig. 2). This could be owing either to an inability of the modified enzyme to bind the nucleotide or to a conformational constraint prohibiting the AMP-induced changes. In kinetic experiments performed with AMP as an inhibitor of the dehydrogenase reaction, similar $K_i$ values were obtained for the native and modified enzymes (3.5 and 3.6 m*M*, respectively). This suggests that modification of the arginine residue does not prevent AMP binding, but blocks the effect of this ligand on the acylation of Cys 149 in the catalytic center.

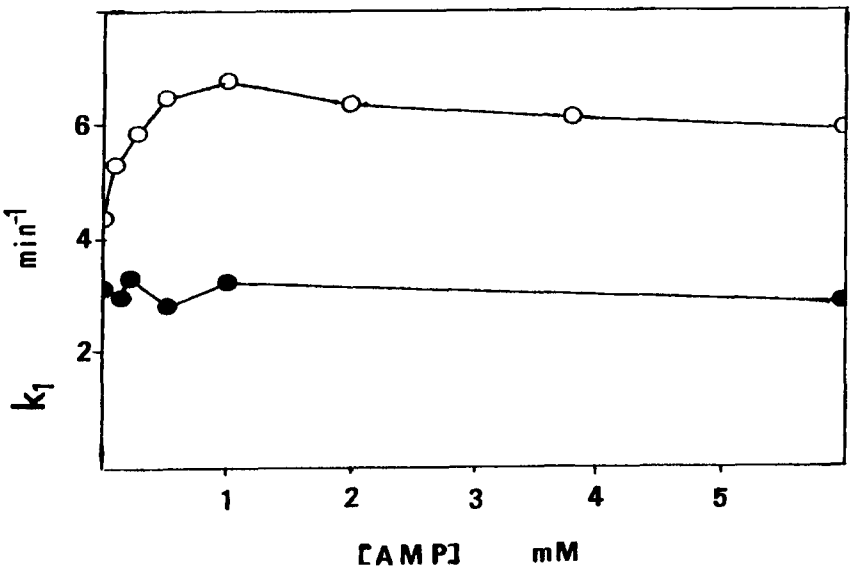


Fig. 2. Effect of AMP on the enzyme acylation rate constant of the *p*-nitrophenyl acetate hydrolysis reaction, catalyzed by the native (○) or 2,3-butanedione modified (●) rabbit muscle GPDH.

Experimental support for the notion that the modified arginine residue locates close to the catalytic subsite of the active center was obtained in our studies on the spectral properties of the enzyme–$NAD^+$ complex. Figure 3 compares the absorption spectra exhibited by the native and modified holo-enzymes. It is seen that the shape of the binary GPDH–$NAD^+$ complex spectrum is significantly altered, on arginine modification, in the region of 310–370 nm. A characteristic property of the native GPDH–$NAD^+$ spectrum (Fig. 3, curve 1) is a broad absorption band centered at 350–360 nm (the Racker band). It is generally accepted that the band is a manifestation of a charge transfer interaction between Cys 149 and the pyridinium moiety of $NAD^+$. The hypothesis was supported in the X-ray study of the binary GPDH–$NAD^+$ complex. The SH- group of the Cys 149 was found near the face of the bound nicrotinamide ring of the oxidized coenzyme (*16*). In the case of the modified enzyme, a progressive decrease in absorbance occurs from 310–370 nm (Fig. 3, curve 2). To evaluate the effect of a charge transfer interaction between Cys 149 and $NAD^+$, the modified GPDH–$NAD^+$ complex was supplemented with 1,3-diphosphoglycerate. This resulted in covalent modification of Cys 149, precluding charge transfer complex formation. Spectrum 3 was then produced, and its subtraction from the spectrum 2 gave the charge-transfer complex spectrum of the modified enzyme (Fig. 3, insert). It is to be noted that the spectrum refers to only two functioning active centers of the asymmetric tetramer of the modified GPDH.

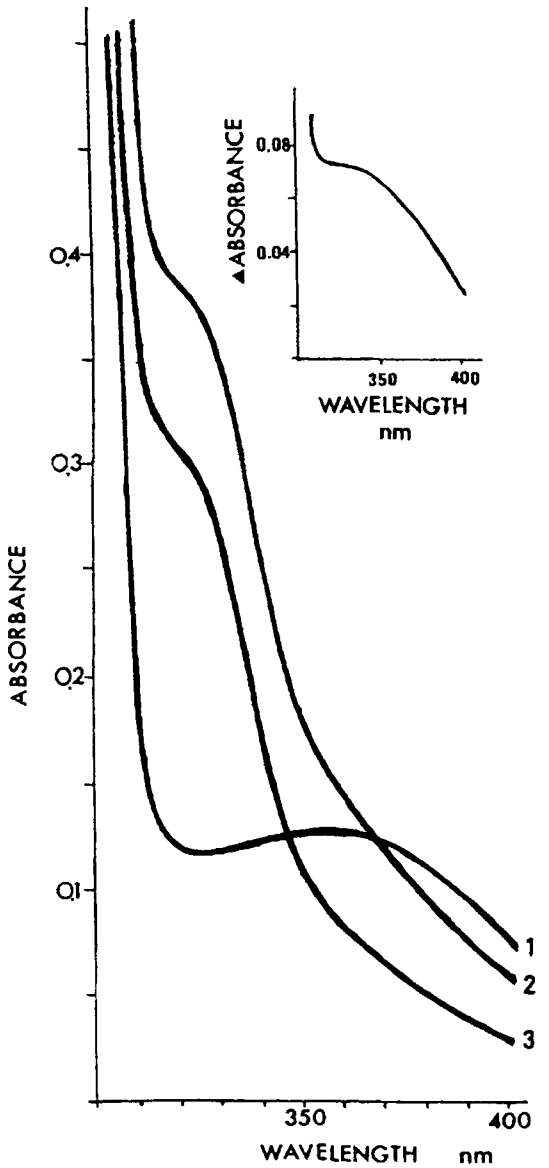


Fig. 3. Absorption spectra of the native and 2,3-butanedione-modified rabbit muscle holo-enzyme. 1, Native holo-GPDH, 32.5 $\mu M$; 2, modified holo-GPDH, 33.0 $\mu M$; 3, the same as 2 plus 240 $\mu M$ 1,3-diphosphoglycerate; insert, the difference spectrum obtained by subtraction of specturm 3 from spectrum 2.

The shape of the charge-transfer complex differs markedly from that of the Racker band exhibited by the native enzyme in complex with $NAD^+$. This is probably because of alterations in the microenvironment of the partners forming the complex, and may indicate that modification of the arginine somehow perturbs a system of intramolecular interactions stabilizing the charge-transfer complex.

## CONCLUSION

The studies on the properties of the enzyme modified at arginine residues provided new information that may complement the knowledge obtained by other experimental approaches. It is pertinent to note that this area of research deserves attention in view of the fact that chemical modification of arginene residues appears to be among the mechanisms of posttranslation protein modification in the cell (*17*).

## ACKNOWLEDGMENTS

We wish to thank A. Kots for his participation in the experiments on the enzyme alkylation with $^{14}$C-iodoacetate. This study was supported by a grant MHB00-MHB300 and NAQ000-NAQ300 from the International Science Foundation and Russian government, by grant 94-04-20215 from the Russian Fund for Fundamental Research, and by a Program "Engineering Enzymology."

## REFERENCES

1. Seydoux, F., Malhotra, O. P., and Bernhard, S. A. (1974), *Crit. Rev. Biochem.* **2,** 227–236.
2. Moras, D., Olsen, K. W., Sabsan, M. N., Buehner, M., Ford, E. G., and Rossman, M. G. (1975), *J. Biol. Chem.* **250,** 9137–9162.
3. Skarzynski, T. and Wonacott, A. J. (1988), *J. Mol. Biol.* **203,** 1097–1118.
4. Mougin, G. A., Corbier, C., Soukri, A., Wonacott, C., Branlant, C., and Branlant, G. (1988), *Protein Eng.* **2,** 45–48.
5. Soukri, A., Mougin, A., Corbier, C., Wonacott, A., Branlant, C., and Branlant, G. (1989), *Biochemistry* **28,** 2586–2592.
6. Corbier, C., Michels, S., Wonacott, A. J., and Branlant, G. (1994), *Biochemistry* **33,** 3260–3265.
7. Kuzminskaya, E. V., Asryants, R. A., and Nagradova, N. K. (1991), *Biochim. Biophys. Acta* **1075,** 123–130.
8. Banas, T., Krotkiewska, B., Marcinkowska, A., and Wolny, M. (1983), *Acta Biochim. Pol.* **30,** 324–334.
9. Kuzminskaya, E. V., Asryants, R. A., and Nagradova, N. K. (1992), *Biochim. Biophys. Res. Commun.* **187,** 577–583.
10. Szewczuk, A., Wolna, E., Wolny, M., and Baranowski, T. (1961), *Acta Biochim. Pol.* **2,** 201–207.
11. Furfine, C. S. and Velick, S. F. (1965), *J. Biol. Chem.* **240,** 844–855.
12. Racker, E. and Krimsky, I. (1952), *J. Biol. Chem.* **198,** 713–743.
13. Wonacott, A. J. and Biesecker, G. (1977), in *Pyridine Nucleotide Dependent Dehydrogenases,* Sund, H., ed,. Walter de Gruyter, Berlin, pp. 140–156.
14. Nagradova, N. K., Asryants, R. A., Benkevich, N. V., and Safronova, M. I. (1976), *FEBS Lett.* **69,** 246–248.
15. Francis, S. H., Meriwether, B. P., and Park, J. H. (1971), *J. Biol. Chem.* **246,** 5427–5432.
16. Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossman, M. G. (1974), *J. Mol. Biol.* **82,** 563–585.
17. Takahara, H., Okamoto, H., and Sugawara, K. (1985), *J. Biol. Chem.* **260,** 8378–8383.